Stereospecific Analysis of High Melting Triglycerides of Bovine Milk Fat and Their Biosynthetic Origin

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Abstract

Fatty acid composition, triglyceride molecular weight distributions, melting ranges, amount of *cis* and *trans* isomers, and stereospecific fatty acid distributions were determined for high melting triglyceride fractions and bovine milk fat free of them. Distributions of fatty acids on the high melting triglycerides differed from distributions of fatty acids in the rest of the milk fat triglycerides. The significance of these results is evaluated in relation to the biosynthetic origin of high melting triglycerides. Stereospecific distributions of these fatty acids support previous speculations that the monoglyceride pathway may play a significant role in biosynthesis of high melting triglyceride in bovine milk fat.

Introduction

Work by deMan (8) demonstrated that there was a difference in physical properties of milk fat before and after interesterification, indicating that the fatty acid distribution on the triglycerides cannot be random. This has been demonstrated by the application of Brockerhoff's methods of stereospecific analysis (6) by Pitas et al. (21) and Breckenridge and Kuksis (3) to milk fat. They found that for intact milk fat there was a nonrandom distribution of fatty acids in all three positions on the triglycerides.

The triglyceride molecular weight distribution for the major triglycerides of milk fat covers a wide range from 26 to 56 fatty acid carbon atoms (18). The distribution for intact milk fat is bimodal with one maximum at C38 and another at C52 (18). High melting triglyceride (HMG) represents a major portion of the high molecular weight triglycerides C48 to C54. Our purpose was to find if HMGs of milk fat have the same stereospecific distribution of fatty acids as the other triglycerides in milk fat.

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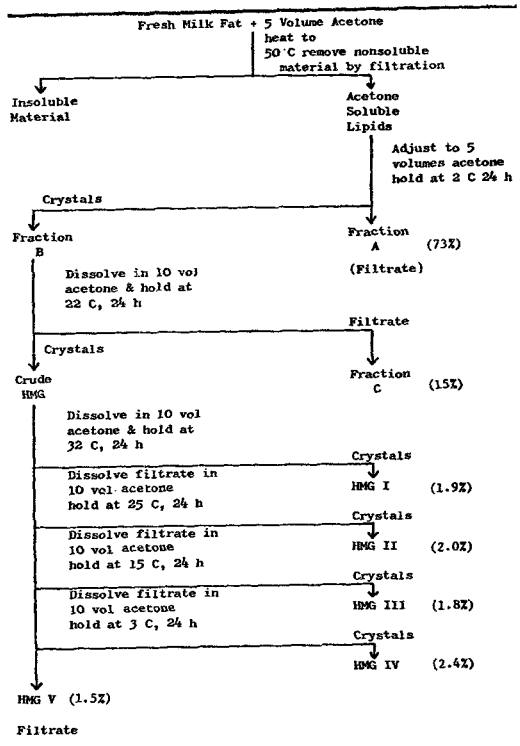
Experimental Methods

Isolation of the high melting glyceride fractions. The initial bovine milk fat was obtained by a 2:1 chloroform:methanol extraction (12) of fresh raw milk from the university herds. The total high melting glyceride fraction was prepared by the method of Patton and Keeney (20). The HMG fraction was separated into five subfractions by progressive fractional crystallization from acetone as in Fig. 1. Separation of fat crystals from liquid fat dissolved in acetone was accomplished by vacuum filtration (23). The volume of acetone at each step in the fractionation scheme was adjusted to at

Fig. 1. Milk Fat Fractionation



least 10 times the volume of the fat to avoid crystallization due to effects of concentration. The percentage yield of initial fat for each fraction is in Fig. 1. Melting ranges of the fractions were determined by differential scanning calorimetry (DSC).

Gas-liquid chromatographic analysis of milk fat fractions. The total fatty acid compositions of the fractions were determined with a Perkin-Elmer 900 gas chromatograph with dual stainless steel columns, 3.8m by 3.2mm packed with 10% EGSS-X by weight on 100/120 mesh Gas Chrom P. Dual flame ionization detectors were used. The columns were temperature programmed from 60 C to 195 C at 6.5 C per min. Methyl esters were prepared by the method of van Wijngaarden (26). Minor component methyl esters were tentatively identified from retention time plots based on isothermal GLC analysis at 150 C (24). Area percent to weight percent correction factors for the programmed runs were determined from repeated injections of a quantitative mixture of fatty acid methyl esters and calculation of correlation factors as by Smith (24) and deMan (8).

The triglyceride molecular weight distributions were determined in dual stainless steel columns (siliconized before packing), 80cm by 3.2 mm packed with 3% OV-1 on 100/120 mesh Gas Chrom Q. Temperature programming was used from 210 C to 340 C at 4 C/min, with a hold of 20 min at 340 C. The carrier gas flow rate was 175 ml/min measured at the column outlets. It was necessary to connect the carrier gas supply directly to the injection port to obtain sufficiently high flow rates. Area percent to weight percent correction factors for triglycerides were determined as for methyl esters of fatty acids. The triglycerides were injected as a 5% solution in chloroform (wt/vol).

Stereospecific analysis of triglycerides. Procedures of Brockerhoff (4, 5, 6) and Sampugna and Jensen (22), with pancreatic lipase to generate representative diglycerides, were used to determine the stereospecific distribution of fatty acids on the triglycerides in different milk fat fractions. The molecular weight specificity of pancreatic lipase (14) was not a problem as milk fat fractions had relatively restricted ranges of triglyceride molecular weights. Lipolysis conditions had to be modified slightly for the HMG I and HMG II samples because they remained solid in the reaction mixture at 37 C. By raising the temperature to 42 C and increasing the amount of enzyme, the HMG II sample remained liquid and was successfully hydrolyzed. Hexane in the reaction mixture was increased from 2 to 4 ml to keep the HMG I sample in solution. The temperature was held at 42 C. These modifications gave satisfactory results without large losses of enzyme activity.

Infrared determination of trans unsaturation. The method of Sreenivasan and Holla (25) was used to determine the concentration of *trans* acids. A standard curve was generated with various concentrations of methyl elaidate (C18:1, *trans*) in CS_2 (wt/wt). Absorbance was measured at 10.38μ. Milk fat fractions were diluted accurately to 15% (wt/wt) solutions in CS_2 and run immediately to avoid changes in concentration due to evaporation of the CS_2. The percent *trans* unsaturation in the sample, expressed as methyl elaidate, was read from the standard curve. The total weight percent unsaturation in the sample was known from the GLC fatty acid analysis. The difference between the total percent unsaturation and the percent *trans* unsaturation was the percent *cis* unsaturation in the sample.

Results

Gas-liquid chromatographic analysis. Total fatty acid compositions of the high and low melting fractions are listed in Table 1. Four fatty acids (C14:0, C16:0, C18:0, C18:1) made up more than 80 mol % of each of the HMG fractions. The simplified fatty acid composition of the HMG fractions indicates that their triglyceride molecular weight distributions should be much narrower than that of whole milk fat. Table 2 shows the triglyceride molecular weight distributions for the milk fat fractions and whole milk fat. Data for whole milk fat are similar to results by Kuksis and Breckenridge (18). Fractions HMG I to IV had a narrower range of major triglyceride species than Fractions A, C, and HMG V. The wide range of the latter was expected since it was a residual material. The four HMG fractions were composed of predominantly high molecular weight triglycerides. Fraction C contained mostly medium and high molecular weight triglycerides while Fraction A and HMG V contained the majority of the lower molecular weight triglycerides. The melting ranges, shown in Table 3, were consistent with the triglyceride molecular weight distributions.

Stereospecific structural analysis. Distributions of fatty acids on triglycerides are in Table 4. Stereospecific distributions were calculated only for fatty acids in concentrations of

TABLE 1. Fatty acid compositions of milk fat fractions: fraction A, fat in filtrate from first crystallization of high melting triglyceride (HMG); fraction C, fat in filtrate from recrystallization of HMG; and HMG fractions I to V, prepared by progressive fractional crystallization from acetone.

Carbon number	Fractions		HMG fractions				
	A	C	I	II	III	IV	V
	Mol % (SD ± 2.5%)						
4:0	3.0	1.1	T[a]	T	...	.1	1.3
6:0	2.6	1.4	T	.1	.2	.2	1.5
8:0	2.4	1.3	T	T	.3	.3	1.2
10:0	5.4	3.8	.2	.3	.9	2.6	3.2
11:0	.5	.2	...	T	T	.1	.3
12:0	5.6	4.3	.8	1.6	3.5	5.5	3.9
12:1	.1	.2	T	T	T	T	.1
12:2	.2	.1	...	T	T	T	.1
13:0	.2	.1	.1	.1	.1	.2	.2
14:BR	.2	.1	T	.1	.1	.1	.1
14:C	12.4	14.0	8.3	12.8	20.0	19.7	13.3
14:1	1.0	.9	.1	.3	.3	.4	.9
14:2	.9	.3	T	T	.4	.8	.3
15:0	1.2	1.2	1.0	1.8	2.0	1.3	1.2
16:BR	.3	.3	.2	.4	.5	.4	.3
16:0	26.6	37.2	44.8	52.7	42.2	39.0	40.9
16:1	1.1	1.0	.5	.5	.6	.5	1.0
16:2	1.3	.8	.4	.9	1.0	.7	.6
17:0	.4	.6	.8	1.0	1.0	.8	.6
18:BR	.4	.2	.2	.2	.1	.1	.2
18:0	7.4	15.3	37.4	22.6	19.4	15.7	13.3
18:1	22.2	13.2	3.1	3.2	6.3	10.1	13.4
19:0	.2	.2	.7	.4	.3	0.2	.2
19:1	.1	T	...	T	T	T	T
18:2	2.9	1.3	.4	.2	.1	.4	1.4
20:0	.1	.1	.6	.4	.3	.2	.1
18:3	1.0	.5	T	T	.1	.3	.5
Longer chain acids	.3	.2	.5	.5	.2	.2	.2

[a] Trace (<.1%).

at least 2 mol % of the total in a given sample (Table 1). Cumulative errors (5) prohibit calculations for minor fatty acids. Concentrations of the major fatty acids chosen for each sample were added and that fraction of the total was multiplied by the observed mole percent of each fatty acid in positions *sn* 1, *sn* 2, and *sn* 3.

From the data in Table 4 for HMG fractions I to IV, C16:0 tends to concentrate in the *sn* 2 position with a medium amount in *sn* 1 and the lowest amount in the *sn* 3 position. Dimick et al. (9) and Wolf and Dugan, Jr. (27) also observed a high concentration of C16:0 on the *sn* 2 position in HMG. The low melting fraction A, 73% of the initial milk fat, had the highest concentration of C16:0 in the *sn* 1 position with a slightly lower concentration in the *sn* 2 position and a considerably smaller amount in *sn* 3. An extreme difference in distribution of C18:0 can be seen in the HMG fractions. In fraction A, C18:0 predominated in *sn* 1 with small amounts in *sn* 2 and *sn* 3. The HMG fractions had C18:0 in high concentration for *sn* 3, a moderate amount in *sn* 1, and the least *sn* 2. There was much lower overall unsaturation in the HMG fractions as compared to low melting glycerides. The major unsaturated fatty acid in HMG, C18:1, was concentrated in the *sn* 3 position with only small amounts in the other two positions. The low melting fraction had a different distribution with the largest amount of C18:1 in the *sn* 1 position. It also had the short chain fatty acids C4:0 and C6:0 almost exclusively in the *sn* 3 position, agreeing with results of Pitas et al. (21).

Infrared analysis. Results of determination of the amount of *trans* unsaturation in the samples are in Table 5. In the three highest melting fractions, greater than 90% of the unsaturation was of the *trans* type while fraction A contained only 29% of its unsaturation in the *trans* form.

Discussion

The fatty acid composition and triglyceride

TABLE 2. Triglyceride molecular weight distributions of milk fat fractions: fraction A, fat in filtrate from first crystallization of high melting triglyceride (HMG); fraction C, fat in filtrate from recrystallization of HMG; and HMG fractions I to V, prepared by progressive crystallization from acetone.

Number of fatty acid carbon atoms	Initial milk fat	Kuksis (18) milk fat	Fractions		HGM fractions				
			A	C	I	II	III	IV	V
	Mcl % (SD ± 1.5%)								
26	.4	.3	.5	.1	...	...	...	...	.1
27	T[a]	...	T	T	...	...	...	...	...
28	1.0	.2	1.3	.3	...	...	...	T	.2
29	T	...	T	T	...	...	...	...	...
30	1.8	1.5	2.2	.6	T	T	T	T	.4
31	.1	...	.1	T	...	...	...	...	...
32	3.3	2.6	4.1	1.2	.1	.1	.1	.1	.8
33	.1	...	.1	T	T	...	T	...	T
34	6.5	5.8	7.9	2.9	.3	.4	.3	.3	2.9
35	.3	...	.4	.1	T	T	T	T	.2
36	11.0	11.8	12.5	8.3	.6	.6	.6	.8	13.1
37	.5	...	.6	.3	T	T	T	T	.4
38	13.1	16.9	14.6	12.0	.5	.6	.7	1.6	14.0
39	.5	...	.6	.2	T	T	T	.1	.4
40	11.1	12.0	12.4	9.9	.3	.5	.8	2.7	10.6
41	.4	...	.4	.3	T	.1	.1	.3	.4
42	7.0	6.7	6.9	9.2	.6	1.4	3.2	8.5	9.6
43	.3	...	.3	.5	.1	.2	.4	.8	.6
44	6.2	4.8	5.3	8.4	2.4	5.9	12.4	17.6	6.8
45	.3	...	.2	.4	.3	.7	1.1	1.4	.4
46	6.5	5.0	5.2	7.8	7.9	17.4	23.5	17.2	5.9
47	.3	...	.2	.4	.7	1.4	2.3	1.5	.4
48	7.2	6.4	5.6	8.8	18.9	25.9	18.6	13.2	9.5
49	.4	...	.3	.6	1.2	1.9	1.7	1.3	.7
50	8.7	9.7	6.2	13.4	30.7	25.3	19.2	15.6	13.1
51	.4	...	.3	.5	1.5	1.2	1.1	1.1	.7
52	8.1	10.2	6.9	10.4	25.9	13.2	10.7	12.6	6.9
53	.1	...	T	T	.4	.2	.5	.3	T
54	4.0	5.9	4.2	3.3	7.0	2.9	2.8	3.1	2.1
55	T	...	T	...	T	T	T	T	...
56	T	T	T	T	.4	.2	.1	.1	...

[a] Trace (< .1%).

TABLE 3. Melting ranges of milk fat fractions.

Sample	Melting range
	(C)
HMG I[a]	53.1 - 61.0
HMG II[a]	51.7 - 57.6
HMG III[a]	43.5 - 51.8
HMG IV[a]	33.4 - 43.8
HMG V[a]	22.6 - 33.7
Fraction A[a]	3.2 - 16.7[b]
Fraction C[a]	37.9 - 47.8

[a] High melting triglyceride (HMG) fractions I to V prepared by progressive crystallization from acetone; fraction A, fat in filtrate from first crystallization of HMG; and fraction C, fat in filtrate from recrystallization of HMG.

[b] Does not include minor melting occurring down to −40 C.

molecular weight distributions for the HMG fractions demonstrate their less complex, highly saturated composition as compared to whole milk fat. This is reflected in the narrow and relatively high melting ranges for the isolated HMG fractions. By stereospecific analysis the fatty acid distribution on the triglycerides of the low melting residual milk fat is markedly different from that of the high melting fractions.

About 35 to 60% of the fatty acids used to synthesize milk fat are obtained from the glycerides of the serum lipoproteins of the blood while the remainder of the fatty acids are synthesized within the mammary cells (1, 2, 13, 19). The glycerol-3-phosphate pathway is the major mechanism of triglyceride synthesis in mammary tissue (2). Synthesis of triglycerides

TABLE 4. Stereospecific analysis of low melting milk fat fractions and high melting triglyceride (HMG) fractions I to V.

Sample	Position	Carbon number 4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2
		Mole %									
Initial milk fat		2.4	2.2	2.0	4.7	5.0	12.7	29.4	9.9	18.9	2.4
Fraction A[a] (Low melting)	*sn* 1	...	...	...	...	1.5	13.3	34.3	14.9	26.0	.7
	sn 2	...	.7	2.5	7.4	7.6	19.4	28.3	3.8	18.1	2.7
	sn 3	9.1	7.3	4.7	8.9	7.5	4.5	17.3	3.7	22.5	5.2
Fraction C[b]	*sn* 1	...	...	...	1.2	2.1	14.5	43.0	17.5	9.4	.7
	sn 2	...	...	...	1.7	3.6	17.6	51.2	7.4	6.5	.5
	sn 3	...	...	...	4.7	3.7	7.8	16.4	21.2	32.3	2.3
HMG I[c]	*sn* 1	...	...	...	...	...	9.6	43.5	38.3	2.1	...
	sn 2	...	...	...	...	...	13.6	64.0	14.6	1.3	...
	sn 3	...	...	...	...	...	.8	35.1	53.3	4.3	...
HMG II[c]	*sn* 1	...	...	...	...	...	18.9	49.7	20.0	2.9	...
	sn 2	...	...	...	...	...	19.0	62.4	8.7	1.4	...
	sn 3	...	...	...	...	...	7.4	39.8	36.2	8.1	...
HMG III[c]	*sn* 1	...	...	...	...	6.7	26.6	35.5	17.8	3.3	...
	sn 2	...	...	...	...	1.3	19.4	56.0	10.9	2.4	...
	sn 3	...	...	...	...	5.2	13.8	19.7	32.7	18.5	...
HMG IV[c]	*sn* 1	...	...	...	2.4	2.9	21.4	41.6	16.4	8.1	...
	sn 2	...	...	...	.6	3.0	16.8	59.0	10.3	3.2	...
	sn 3	...	...	...	5.1	9.9	15.2	17.0	22.6	23.0	...
HMG V[c]	*sn* 1	...	...	1.4	2.6	3.0	12.6	52.2	14.3	3.4	...
	sn 2	...	...	.4	2.4	3.7	19.1	49.6	6.5	7.7	...
	sn 3	...	...	1.7	6.0	6.5	6.8	14.9	18.4	35.2	...

[a] Fat in filtrate from first crystallization of HMG.
[b] Fat in filtrate from recrystallization of HMG.
[c] Fractions I-V prepared by progressive crystallization from acetone.

TABLE 5. Percent *trans* unsaturation in high melting triglyceride (HMG) and low melting fractions of milk fat.

Sample	Total unsaturation[a]	*Trans*[b]	*Trans* as % of total[c]
	Weight %		
HMG I[d]	4.6	4.6	100
HMG II[d]	6.5	6.6	100
HMG III[d]	10.5	9.6	92
HMG IV[d]	15.3	8.3	55
HMG V[d]	18.9	8.4	44
Fraction A[e]	31.6	9.3	29

[a] Determined by gas liquid chromatography (GLC), ± 2.5% of values shown.
[b] Determined by Infrared (IR) Spectroscopy, ± 2.0% of values shown corrected by a factor of 1.0/1.09 to account for difference between IR and GLC determination on the same standard.
[c] (*Trans*/total unsaturation) × 100/(Total unsaturation) = *Trans* as a % of total.
[d] Fractions I-V prepared by progressive crystallization from acetone.
[e] Fat in filtrate from first crystallization of HMG.

by this pathway involves the acylation of a diglyceride precursor with endogenously synthesized fatty acids (16). The fatty acids endogenously synthesized from acetate and β-hydroxy butyrate generally have a chain length of 16 carbons or less with relatively large quantities of short chain fatty acids being produced (11). Diglyceride precursors would be predominantly of the 1, 2 diglyceride type based on our observations and previous observations (3, 21) of large amounts of endogenously synthesized short chain fatty acids in the *sn* 3 position of milk fat triglycerides.

The other pathway of triglyceride synthesis uses monoglycerides derived from incomplete hydrolysis of triglycerides associated with the low density lipoprotein fraction of the blood. Some of these monoglycerides are able to pass through the cell membrane (9, 16). These monoglycerides then can have fatty acids esterified on the other two positions to form a triglyceride. Any diglycerides or intact triglycer-

ides in the blood are probably too large to pass through the mammary cell membrane.

From this and data of stereospecific analysis in Table 4, it is possible to postulate the origins of low melting and high melting glycerides. Dimick et al. (9) said a "blood lipid source (conceivably 2-monoglycerides) appears to be influencing the 2-position of the milk fat triglycerides."

Bickerstaffe (2) discussed milk fat synthesis and said "the monoglyceride pathway is probably the minor route to triglycerides in the mammary tissue." Therefore, most of the milk fat triglycerides are probably synthesized via the glycerol-3-phosphate pathway.

The medium to low melting triglycerides are more than 75% of milk fat and would be made predominantly via the glycerol-3-phosphate pathway. The stereospecific analysis for whole milk fat (Table 6) and the low melting fraction A indicates that there is no preference for placement of palmitic acid in the *sn* 2 position of the majority of the triglycerides. The palmitic acid distribution is almost equally high in the *sn* 1 and *sn* 2 positions of most of the milk fat triglycerides or, in other words, the lower melting glycerides.

The high melting glycerides do have palmitic acid esterified predominantly in the *sn* 2 position of the triglycerides. If the high melting glycerides originate via the monoglyceride pathway, then the blood glycerides associated with the low density lipoprotein fraction should contain considerable amounts of palmitic acid in the *sn* 2 position. Dimick et al. (10) found high concentrations (up to 76%) of palmitic acid in the *sn* 2 position of triglycerides associated with low density lipoproteins in cow and goat blood serum. The large quantities of stearic acid in the *sn* 3 position of the triglycerides in the HMG fractions suggests that stearic acid was attached shortly after the monoglyceride entered the cell from the blood. Kinsella thought that the overall process of fatty acid absorption from the blood involves adsorption of fatty acid to the cell membrane, activation of the fatty acid to coenzyme A or an acyl carrier protein derivative, transport across the plasma membrane, and rapid esterification of entering activated fatty acids (15). This leads to speculation that some monoglycerides are acylated all the way to triglycerides before they reach the inner surface of the microsomal layer where they would normally pick up an endogenously synthesized short chain fatty acid in the *sn* 3 position. Such triglycerides of high molecular weight would make up most of the HMG fraction of milk fat. It is also likely that some high melting triglycerides come from the glycerol-3-phosphate pathway. These would be the triglycerides with palmitic or myristic acids in the *sn* 3 position.

The longer chain fatty acids, the C18 fatty acids, and part of the C16 fatty acids enter the mammary cell from the blood. Some of the C18:1 fatty acids (probably only *cis* isomers) are made from exogenous stearic acid, C18:0, by the action of the enzyme stearyl desaturase. Kinsella (17) found that stearyl desaturase activity was associated only with the microsomal fraction of the subcellular components of mammary cells. Substantial quantities of C18:1 can also enter the mammary cell from the blood. Bickerstaffe (2) demonstrated that both oleic and elaidic acid are taken up from the blood by the mammary cells and incorporated into the milk fat to the same extent. Also considerable quantities of elaidic acid can be in the blood. This is due to the hydrogenation of fatty acids in the rumen resulting in conversion of some of the *cis* unsaturation to *trans* and the absorption of these fatty acids from the intestine into the blood stream.

Infrared data in Table 5 indicated that the three highest melting fractions contain almost exclusively the *trans* isomer of C18:1. The stereospecific analysis, in Table 4, shows that the C18:1 in the three highest melting fractions is predominantly in the *sn* 3 position. Stearic acid and elaidic acid are similar in physical properties because the *trans* double bond of elaidic acid causes little distortion in

TABLE 6. Stereospecific analysis of bovine milk fat reported by Pitas et al. (21).

Sample	Position	Carbon number 4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2
		Mole %									
Whole milk fat	Overall fatty acid composition	11.3	4.8	2.3	4.2	4.0	11.5	27.1	10.4	21.1	1.4
Whole milk fat	*sn* 1	5.0	3.0	.9	2.5	3.1	10.5	35.9	14.5	20.6	1.2
	sn 2	2.9	4.8	2.3	6.1	6.0	20.4	32.8	6.4	13.7	2.5
	sn 3	43.3	10.8	2.2	3.6	3.5	7.1	10.1	4.0	14.9	—0.46

the linear carbon chain. It appears that the *trans* isomer of C18:1 is distributed on the triglycerides almost as if it were a C18:0.

The amount of C18:1 fatty acids seems to be about equally high in the *sn* 1 and *sn* 3 position and lower in the *sn* 2 position in the low melting fraction A (Table 4). The total C18:1 in fraction A is approximately 70% oleic acid and 30% elaidic acid. If the elaidic acid was distributed in this fraction similar to stearic acid, then most of the elaidic acid in fraction A should be in the *sn* 1 position. This speculation should be confirmed by infrared analysis of the lysophosphatide fraction in the stereospecific analysis scheme.

Conclusion

The stereospecific fatty acid composition of the HMG fractions of milk fat seemed to support the previous evidence (10) for a monoglyceride pathway of milk fat synthesis in addition to the glycerol-3-phosphate pathway. The high concentration of stearic acid and elaidic acid in the *sn* 3 position of the HMG fractions indicated that the triglyceride may have been formed completely before it reached the inner surface of the microsomal layer. This information, coupled with the preferred placement of palmitic acid in the *sn* 2 position of the HMG triglycerides, suggests that there was something influencing the *sn* 2 position of the HMG triglycerides that had little effect on the rest of the milk fat triglycerides. The entrance of 2-monoglycerides from the blood stream, with predominantly palmitic acid in the *sn* 2 position, could account for the preferential placement of palmitic acid in the *sn* 2 position of the HMG. This explanation is based on observed compositional data and should be investigated further by radiotracer techniques for confirmation.

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