STRUCTURE OF HIGH-MELTING GLYCERIDES FROM THE MILK FAT-GLOBULE MEMBRANE

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ABSTRACT

STRUCTURE OF HIGH-MELTING GLYCERIDES
FROM THE MILK FAT-GLOBULE MEMBRANE

by Don P. Wolf

This study was undertaken to determine the structure of high-melting glycerides associated with the milk fat-globule membrane.

The procedure involved digestion of the triglyceride with pancreatic lipase, chromatographic separation of the fatty acids and glycerides and gas-liquid chromatography of the fatty acid esters from the separated fractions.

Definition of location of individual fatty acids within the membrane high-melting glycerides indicated that the beta positions of these glycerides were occupied primarily by a saturated fatty acid of 14, 16 or 18 carbon atoms.

The trisaturated glyceride content of membrane high-melting glyceride was found to be 71.2% while, those isomeric forms of disaturated-monounsaturated and monosaturated-diunsaturated glycerides which contained a saturated fatty acid in the beta position were found to predominate.

Calculation of triglyceride types and isomeric forms indicated that, while random distribution was found on the basis of saturated and unsaturated, the individual fatty acids were not randomly distributed in the triglycerides under study.

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By

Don P. Wolf

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INTRODUCTION

Considerable research in the last few years has been devoted to the nature of the fat globule membrane existing in whole milk. However, the natures of the proteins and lipids in the complex structure composing this membrane are not well characterized. Interest has centered around a high-melting glyceride fraction isolated from several sources but presumed to be associated with the milk fat membrane. Present theories suggest that this fraction may serve as a link between the fat globule and the membrane.

The purpose of this study was to determine the structure of this high-melting glyceride, which is insoluble in 95% ethanol at room temperature, in order to contribute to the elucidation of the glycerides associated with the fat-globule membrane.

LITERATURE REVIEW

High-Melting Glyceride

The lipid-protein nature of the membrane present at the fat-plasma interface in whole milk has been recognized for years. Palmer and Weise (1933) were the first to isolate high-melting glycerides (HMG) from the membrane. Rimpula and Palmer (1935) isolated HMGs from the membrane of artificial as well as natural creams. They postulated that the fatty acid residues of the membrane phospholipids were attached to this HMG with almost chemical affinity. Jenness and Palmer (1945) isolated high-melting triglycerides of similar properties from butterfat (4.5% of total lipid), washed-cream buttermilk extracts and washed-cream serum extracts (37.4%) by crystallization from ethanol. They reported the following physical properties for these fractions: iodine value 5.0-7.1, saponification equivalent 198.8-204.0 and melting point 52-53° C. Jenness and Palmer (1945) found that the characteristics of the HMG obtained from butteroil by crystallizing from ethanol were independent of the composition of the butteroil. In order to explain the high concentrations of HMG found in butter serum, Jenness and Palmer (1945) proposed that during churning some of the protein-phospholipid and phospholipid-HMG linkages are broken. The protein-phospholipid complex was then found to be relatively richer in protein than the original membrane while the complex of the butter serum was relatively richer in phospholipid. Upon melting the butter, the phospholipids continue to cling to some of the HMG molecules pulling them into the serum thus accounting for the higher proportions of HMG in butter serum.

The hypothesis of Rimpula and Palmer (1935) was substantiated by Jenness and Palmer (1945) when they found that ether extracted only small amounts of HMG from unconcentrated butter serum indicating that this fraction was still "bound" by the membrane. Patton and Keeney (1958) reported the isolation of an acetone-insoluble HMG from the membrane. The characteristics of this fraction and the ethanol-insoluble glyceride fraction obtained by Thompson, Brunner and Stine (1959) were similar in that stearic and palmitic acids were the principal fatty acid components. The iodine values reported by these workers showed no agreement. The absence of glycerides in the ethanol soluble fraction led Thompson et al. (1959) to suggest that the entire membrane triglyceride is HMG. Keeney (1961) reported the presence of distearyl triglycerides in an acetone-insoluble high-melting glyceride from milk fat. However, he found that none of the triglycerides from this fraction contained more than 50% stearic acid. Since any tristearin present would surely be a constituent of the HMG fraction, it was deduced that milk fat contains no tristearin.

Several good reviews have been made on the fatty acid composition of milk fat: Jack and Smith (1956) and Shorland and Hansen (1957). The work on fatty acid composition of milk fat that has been accomplished in the past few years was reviewed by Herb, Magidman, Luddy and Riemenschneider (1962). From their own research they reported the identification of at least 60 fatty acids in milk fat. This list included several acids (odd-numbered, monoethanoid from C15-23) not previously reported. Twenty-seven minor fatty acids were found each in a concentration of less than 0.1%.

Boatman, Decoteau and Hammond (1961) and Eshelman, Manzo, Marcus, Decoteau and Hammond (1960) using the mercaptoacetic acid method found milk fat to contain from 21.5 to 32.0% by weight trisaturated glyceride. Their results showed no preferential selection or exclusion of any of the major fatty acids from the trisaturated glycerides and the percentages agreed with the amount calculated by random distribution.

Pancreatic Lipase

Pancreatic lipase provides a useful method for determining the structure of glycerides because of its specificity for hydrolyzing the ester linkages at the one and three positions of triglycerides. Studies on the specificity of this enzyme have been made by a number of workers, notably by Mattson and Beck in America (1955, 1956) and Savary and Desnuelle (1955, 1956) in France. Enzymatic hydrolysis of synthetic triglycerides of known structure has verified the specificity of this enzyme.

Desnuelle (1961) reported that shorter, saturated fatty acids are liberated more rapidly than longer ones and that saturated acids from C18 down to C12 and the C18 monounsaturated fatty acids are split off at similar rates. He also found that pancreatic lipase acts exclusively on emulsified esters for he observed an increase in activity when emulsions began to form and a decrease or the absence of activity when the substrate was in solution. All workers agreed that the hydrolysis of triglycerides by pancreatic lipase proceeds in a stepwise fashion from triglyceride to 1,2-diglyceride to 2-monoglyceride.

Pancreatic lipase has been used extensively in the determination of the structure of triglycerides. Youngs (1961) described a method

for the quantitative determination of the six types of glycerides found in fats. VanderWal (1960), Mattson and Lutton (1958) and Patton, Evans and McCarthy (1960) have done similar work on the structure of milk fat employing gas-liquid chromatography for analyzing the hydrolyzed products. Patton et al. (1960) found a higher concentration of C10, C12 and C14 saturated and C14 and C16 monounsaturated acids and a lower concentration of C18 acids in the monoglycerides after digestion with pancreatic lipase. Ast and VanderWal (1961), from a study of the distribution of saturated and unsaturated fatty acids of butterfat, concluded that butterfat is another of the group of fats in which saturated and unsaturated acids become associated as S_3, S_2U, SU_2 and U_3 in proportions which can be specified, at least approximately, by application of the laws of probability operating freely or with some restriction.

Jensen, Sampugna and Gander (1961) used milk lipases, which are also specific for the one and three positions of glycerol, to determine the composition of butteroil. Their studies revealed lower concentrations of C4, C18 saturated and C18 monounsaturated acids and higher amounts of C14 acids in the monoglycerides after hydrolysis. Kumar, Pynadath and Lalka (1960) used pancreatic lipase to establish that butyric acid is located at the alpha positions in milk fat.

Thin-Layer Chromatography

Thin-layer adsorption chromatography (TLC) on silicic acid has been used for the resolution of a wide variety of lipids. The method as applied to lipids remained in obscurity until Stahl, Schroter, Kraft and Renz (1956) described equipment and procedures for the preparation

of chromatoplates and demonstrated the potential usefulness of TLC in the fractionation of substances other than terpenes. Several reviews of the literature on the general application of TLC have been made: Demole (1958), Stahl (1958) and Mangold (1961).

Thin-layer chromatography has been applied to mixtures of mono-, di- and triglycerides by several workers. Jensen et al. (1961), who hydrolyzed milk fat with milk lipases, used TLC in separating glycerides from a silica gel column. Privett and Blank (1961) developed a micro-method for the determination of component mono-, di- and triglycerides. Their method included the ozonization of double bonds and the catalytic reduction of the ozonides followed by separation and quantification of the glyceryl residues by TLC. Malins and Mangold (1960, 1960) also reported on the separation of mono-, di and triglycerides on TLC. In addition, they separated the methyl esters of a C18 fraction derived from menhaden oil by reversed-phase-partition chromatography on siliconized silicic acid plates.

EXPERIMENTAL PROCEDURE

The cream used throughout this study was supplied by the Michigan State University Creamery from a mixed herd source.

Figure 1 represents schematically the procedure employed in isolating the HMG from the milk fat-globule membrane.

The steps followed in this experiment to determine glyceride structure were: (1) digestion of the triglyceride with pancreatic lipase, (2) isolation of the digestion products, and (3) determination of the fatty acid composition of these products.

Pancreatic Lipase Digestion

The conditions of digestion were selected from several procedures previously reported (Mattson and Volpenhein, 1961 and Ast and VanderWal, 1961).

The digestion mixture consisted of 0.2-0.5 g. of triglyceride, 20 ml. of distilled water, 0.5 ml. of 45% aqueous solution of $CaCl_2$, 0.2 ml. of a 1% aqueous solution of bile salts (sodium taurocholate) and 100 mg. of pancreatic lipase (Mann Research Laboratories, pork pancreas-crude). Digestion was carried out at 40° C. with continuous agitation. The pH was maintained at 8 by periodic additions of 0.1 N NaOH and the digestion was allowed to proceed for five minutes.

At the end of the hydrolysis period, 5 ml. of 6N HCl and 15 ml. of ethanol were added. The lipids were then recovered by extraction with petroleum ether. The petroleum ether solution was washed with water, dried with sodium sulfate, and the solvent removed under vacuum.

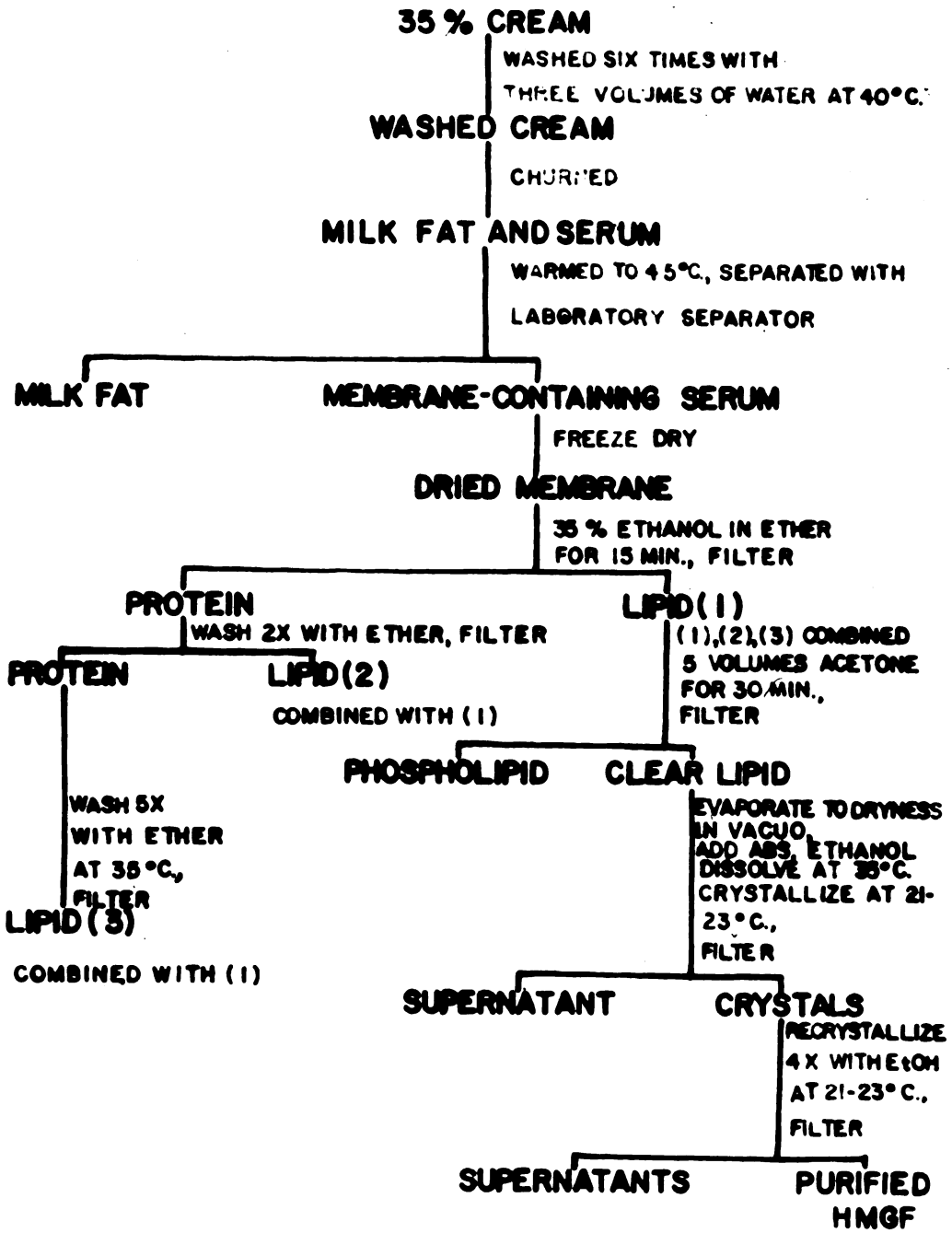


Fig. 1. Schematic isolation procedure for obtaining the high-melting glyceride fraction from the milk fat-globule membrane.

Column Chromatography

The neutral glycerides and fatty acids liberated during digestion were separated by adsorption column chromatography on Florisil according to the method of Carroll (1961). The free fatty acids, tri-, di- and monoglycerides were eluted in a stepwise fashion employing ethyl ether, hexane, methanol and acetic acid as solvents. The column eluate was collected in 25-ml. portions, the solvent removed and the purity of each fraction was ascertained on thin layers of silicic acid as described below. Figure 2 represents a typical separation of glycerides by thin-layer chromatography. The acetic acid employed during elution of the free fatty acids was removed from the sample by washing with water.

Thin-Layer Chromatography

The plates for thin-layer chromatography were prepared as outlined by Mangold (1961).

TLC was employed directly to separate the digested lipid mixture. Samples were dissolved in a minimal amount of chloroform and streaked on the thin-layer silicic acid plates. Solvent systems of 50:50:1 and 10:90:1, ethyl ether:petroleum ether:acetic acid were employed and the plates were developed for 30-45 minutes. The resulting glyceride bands were made visible under ultraviolet light by spraying with dichlorofluorescein. The outlined bands, defining the separate fractions, were recovered by scraping off the glass plate and extracting with petroleum ether.

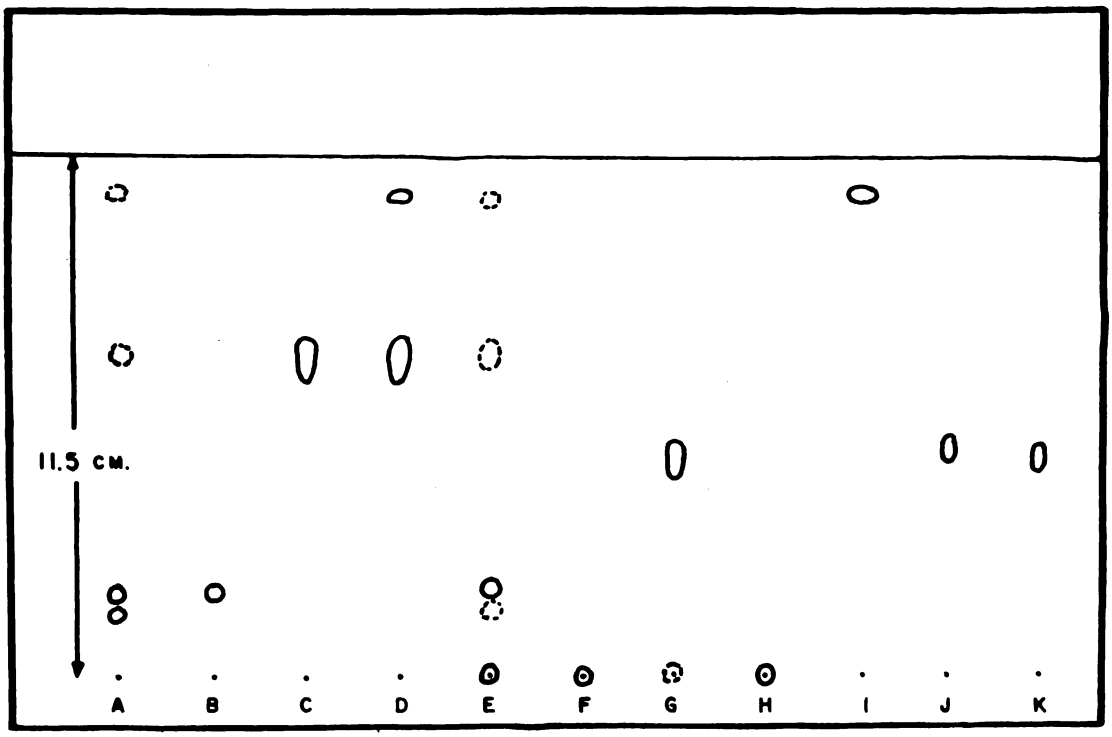


Fig. 2. Thin-layer adsorption chromatography of lipid classes on Silica Gel. Solvent: Petroleum ether-ethyl ether-acetic acid, 90/10/1, v/v/v. Development time: 40 min. Indicator: Dichlorofluorescein. Amounts: approx. 20λ each. a) diglyceride from Florisil column, b) cholesterol c) tripalmitin, d) triglyceride from Florisil column, e) monoglyceride from Florisil column, f) monoglyceride, g) fatty acids from Florisil column, h) acetic acid, i) cholesterol acetate, j) myristic acid, k) capric acid.

Gas-Liquid Chromatography

Methyl esters of the neutral glyceride fatty acids were prepared according to the base-catalyzed interestification described by Smith and Jack (1954). The free fatty acids were esterified by refluxing in methanolic HCl (Youngs, 1961) or in methanol and sulfuric acid (Trammel and Janzen, 1961).

An Aerograph Model A-90-C Gas Chromatograph equipped with tungsten hot wire thermal conductivity detectors was employed in conjunction with a Leeds and Northrup Type G Speedomax recorder having a 5 mv. scale span and chart speed of 2 min./inch. Ten foot, one-quarter inch (O.D.), copper gas chromatographic columns packed with diethylene glycol succinate were employed for the resolution of fatty acid methyl esters C4-C18.

Operating conditions were as follows: Oven temperature-180° C., injector temperature-240° C., helium flow rate-75-100 cc./minute and detector current-265 ma.

Quantitative analysis was achieved by measuring the areas of the peaks with a planimeter. Unresolved peaks were separated for measurement by drawing the shortest possible perpendicular line from the recorder tracing to the baseline of the chromatogram. The area percentage for the major fatty acids, based on the total area of the major fatty acids, was computed. Unidentifiable areas and minor fatty acid areas were not included in this calculation.

EXPERIMENTAL RESULTS

The fatty acid compositions listed in Tables I-V were expressed quantitatively as percentages of the major peak areas of the gas chromatograms. The fatty acids were identified by 18:1 or similar designation, the first number referring to the number of carbons in the chain and the second the number of double bonds.

The results of the fatty acid analyses of butteroil triglycerides and the diglycerides, monoglycerides and free fatty acids resulting from lipolytic action are presented in Table I. Butteroil, as used in this text, refers to the milk lipid not associated with the fat-globule membrane. The data indicate that 10:0, 12:0, 14:0 and 16:0 existed in higher concentrations in both the diglycerides and monoglycerides, except for 10:0 in the diglycerides. In contrast, 18:0 and 18:1 were found in lower concentrations in the partial glycerides. These results were substantiated by the free fatty acid analyses which indicated a higher than random concentration of 18:0 and 18:1 esterified at the alpha position of butteroil.

The data presented in Table I agree well with those of McCarthy, Patton and Evans (1960), Patton et al. (1960) and Jensen et al. (1961).

These results from the lipolysis of the HMG isolated from butteroil are expressed in Table II. Increased quantities of 10:0, 12:0, 18:1 and 18:2 and decreased quantities of 16:0 and 18:0 were found in the free fatty acid fraction. The value for 14:0 while showing an overall increase, was not as consistent.

TABLE I

Fatty acid compositions of butteroil triglycerides and the diglycerides, monoglycerides and free fatty acids resulting from lipolytic action

Acid[a]	Triglyceride[b]	Diglyceride[b]	Monoglyceride[c]	Free fatty acids[c]
	Area%			
8:0	1.1	0.8	0.0	2.8
10:0	3.6	2.9	3.8	2.2
12:0	6.0	6.4	7.0	3.4
14:0	15.4	18.3	22.7	10.6
16:0	34.5	37.4	39.0	34.3
18:0	9.3	8.2	7.3	16.3
18:1	26.0	22.4	17.9	28.2
18:2	3.8	2.4	1.7	2.2
TOTAL S	70.2	75.2	80.4	69.6
TOTAL U	29.8	24.8	19.5	30.3

[a] The first figure designates the number of carbons and the second the number of double bonds.

[b] Average of four samples.

[c] Average of three samples.

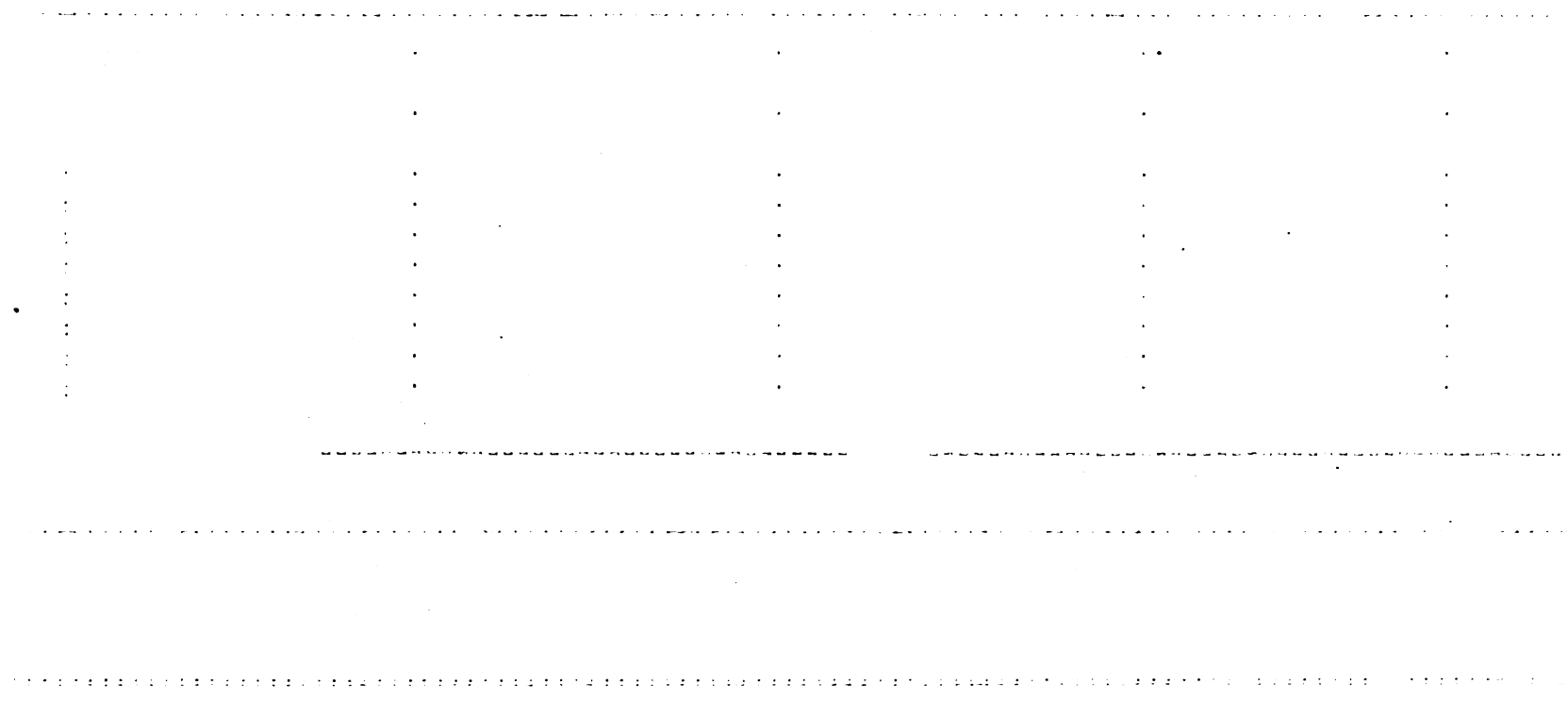


TABLE II

Fatty acid compositions of butteroil high-melting triglycerides and the free fatty acids resulting from lipolytic action[a]

Acid	Triglyceride	Free fatty acids
	Area %	
10:0	1.7	2.1
12:0	3.6	5.5
14:0	21.0	22.0
16:0	45.7	41.7
18:0	14.2	11.7
18:1	12.1	14.8
18:2	1.4	2.2
Total S	86.5	82.9
Total U	13.5	17.0

[a] Average of three samples

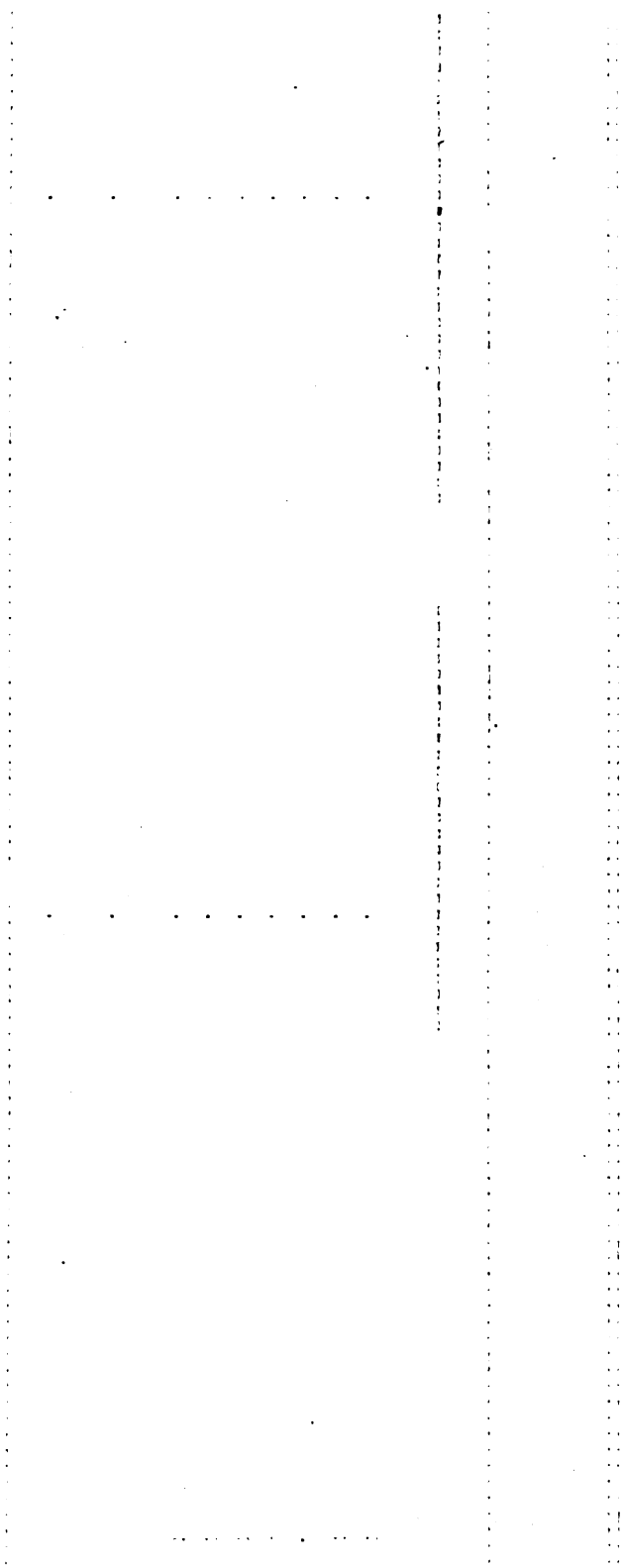


Table V represents an average of six individual determinations of glyceride structure (Tables III-IV) of HMG from the fat-globule membrane. In most respects the data were similar to those exhibited by the HMG of butteroil indicating a higher than random concentration of 16:0 and 18:0 in the beta position of the triglyceride. In contrast to the results with butteroil HMG, the concentration of 14:0 decreased in the free fatty acid fraction of lipolyzed membrane HMG.

The compositions of butteroil HMG and membrane HMG show striking dissimilarities in the saturated fatty acids myristic and stearic. The melting point for butteroil HMG was found to be from 5-8° C. below the 52-53° C. reported by Thompson et al. (1959) for membrane HMG (see Figures 3 and 4).

Using data obtained from pancreatic lipase hydrolysis, the triglyceride types and isomeric forms were computed for the fats studied, by the method of VanderWal (1960). The results are shown in Table VI. Comparison of the calculated values for triglyceride types with those predicted by random distribution indicate that the HMGs, as well as butteroil, are among that group of fats in which saturated (S) and unsaturated (U) become associated as S_3, S_2U, SU_2 and U_3 in proportions which can be specified, at least approximately, by application of the laws of probability, see Table VII.

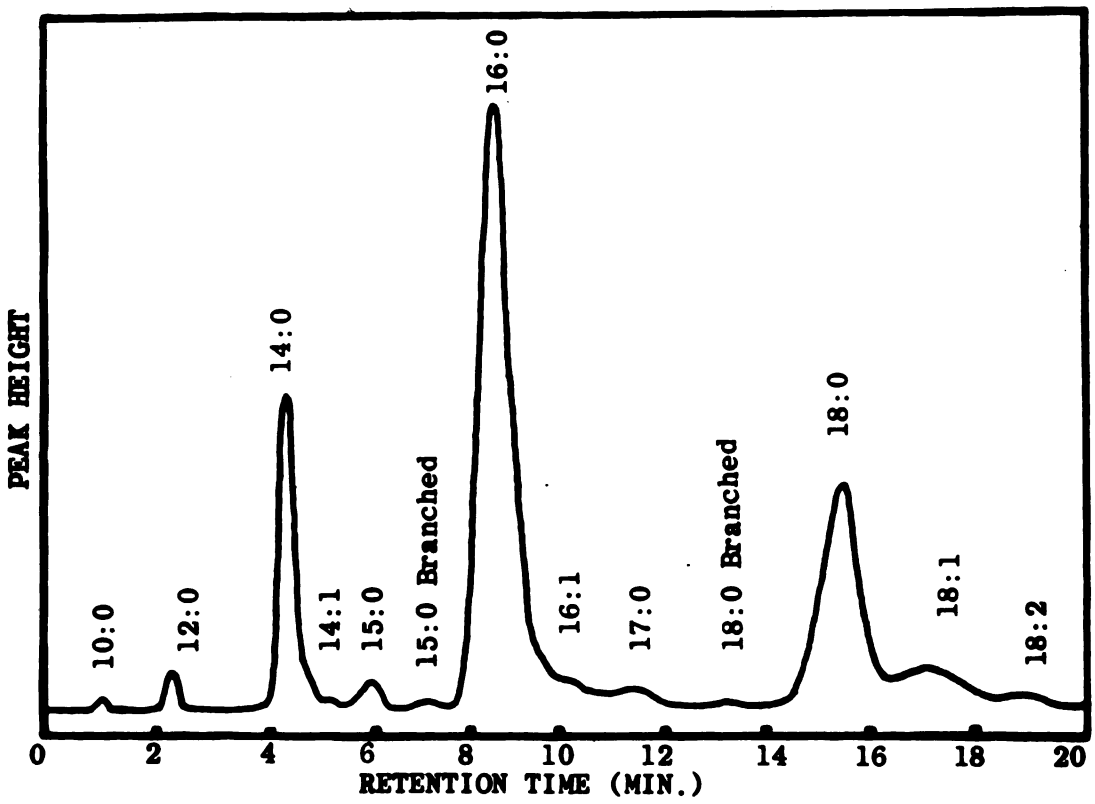


Fig. 3. Gas chromatogram of fatty acid methyl esters obtained from membrane HMG preparation No. 5.

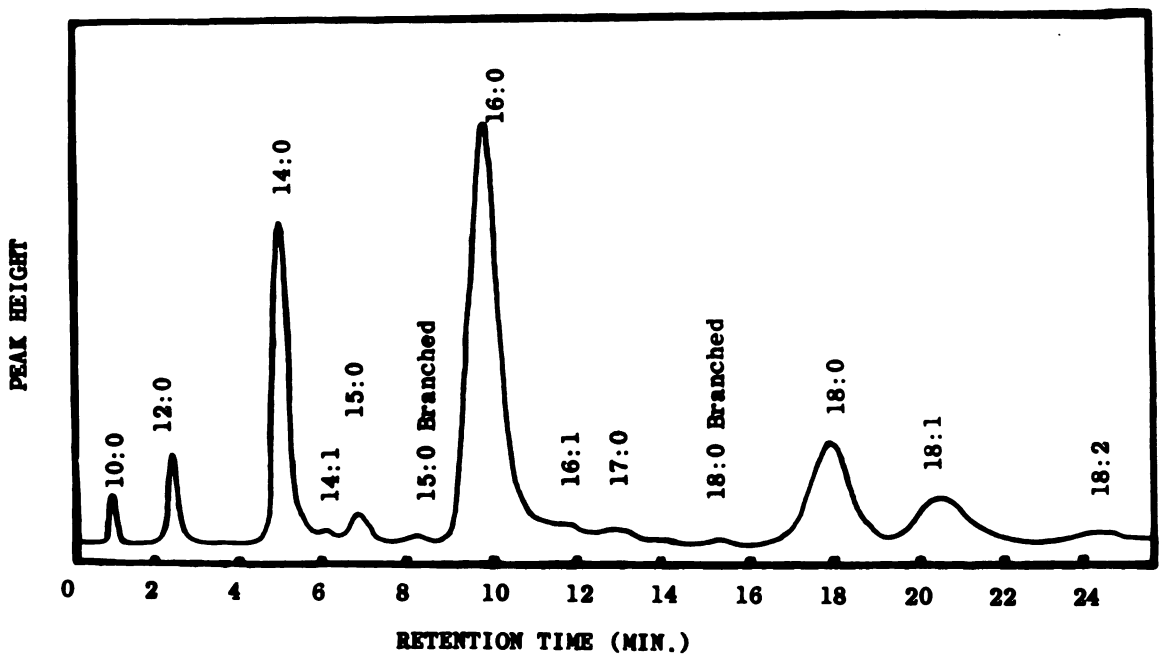


Fig. 4. Gas chromatogram of fatty acid methyl esters obtained from butter oil HMG preparation No. 2.

TABLE III

Fatty acid compositions of membrane HMG and the products of lipolysis from HMG preparations 1, 2 and 3

	HMG preparation No. 1		HMG preparation No. 2		HMG preparation No. 3	
Acid	TG	FFA	TG	FFA	TG	FFA
	Area %					
10:0	1.1	2.3	1.1	2.1	0.3	0.6
12:0	1.6	3.9	2.8	6.3	1.1	1.9
14:0	11.1	13.2	15.3	14.6	13.7	14.3
16:0	45.5	40.8	44.3	41.5	48.8	44.7
18:0	29.1	22.8	23.4	18.8	28.5	23.2
18:1	10.1	14.9	12.0	18.8	6.0	13.1
18:2	1.6	2.3	1.8	2.0	1.6	2.2
Total S	88.3	79.9	86.8	79.2	92.2	82.6
Total U	11.7	20.1	13.1	20.8	7.7	17.4

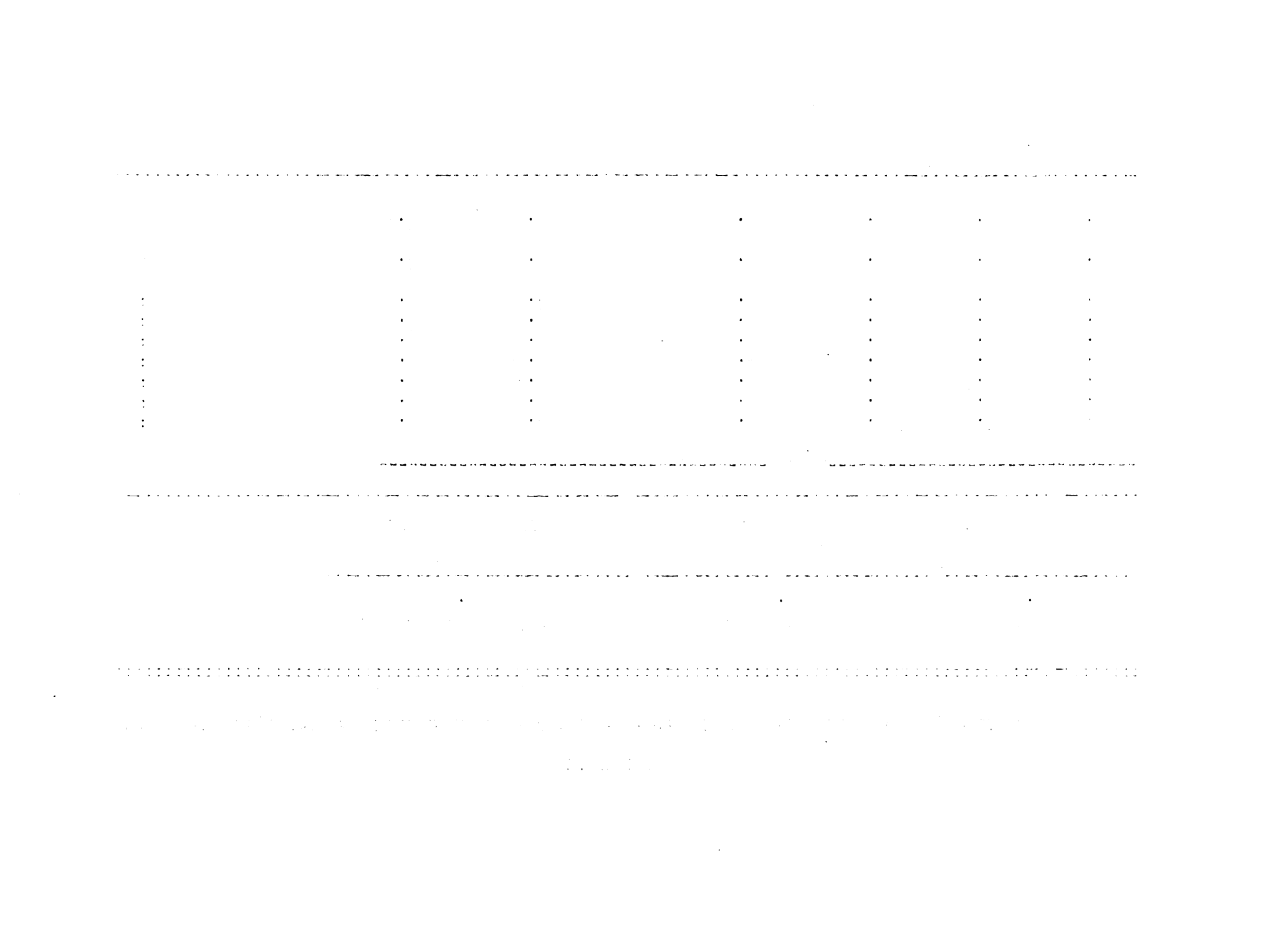


TABLE IV

Fatty acid compositions of membrane HMG and the products of lipolysis from HMG preparations 4, 5 and 6

	HMG preparation No. 4			HMG preparation No. 5			HMG preparation No. 6		
Acid	TG	DG	FFA	TG	DG	FFA	TG	DG	FFA
	Area %								
10:0	2.4	1.5	4.3	1.0	0.3	1.8	1.1	1.0	2.2
12:0	4.7	3.5	8.7	3.4	3.1	4.6	2.0	2.3	4.5
14:0	15.3	19.3	13.0	15.1	17.7	14.2	14.8	16.3	10.8
16:0	43.5	47.2	36.8	48.7	45.1	38.3	47.1	49.8	43.2
18:0	20.0	19.3	18.4	24.4	26.0	18.3	24.5	20.9	23.1
18:1	12.9	9.3	15.4	6.2	7.8	10.6	9.6	8.7	13.9
18:2	1.2	0.0	3.3	1.2	0.0	3.3	1.0	1.0	2.4
Total S	85.9	90.7	78.3	92.6	92.2	86.2	89.4	90.3	81.7
Total U	14.1	9.3	21.7	7.4	7.8	13.8	10.6	9.7	18.3

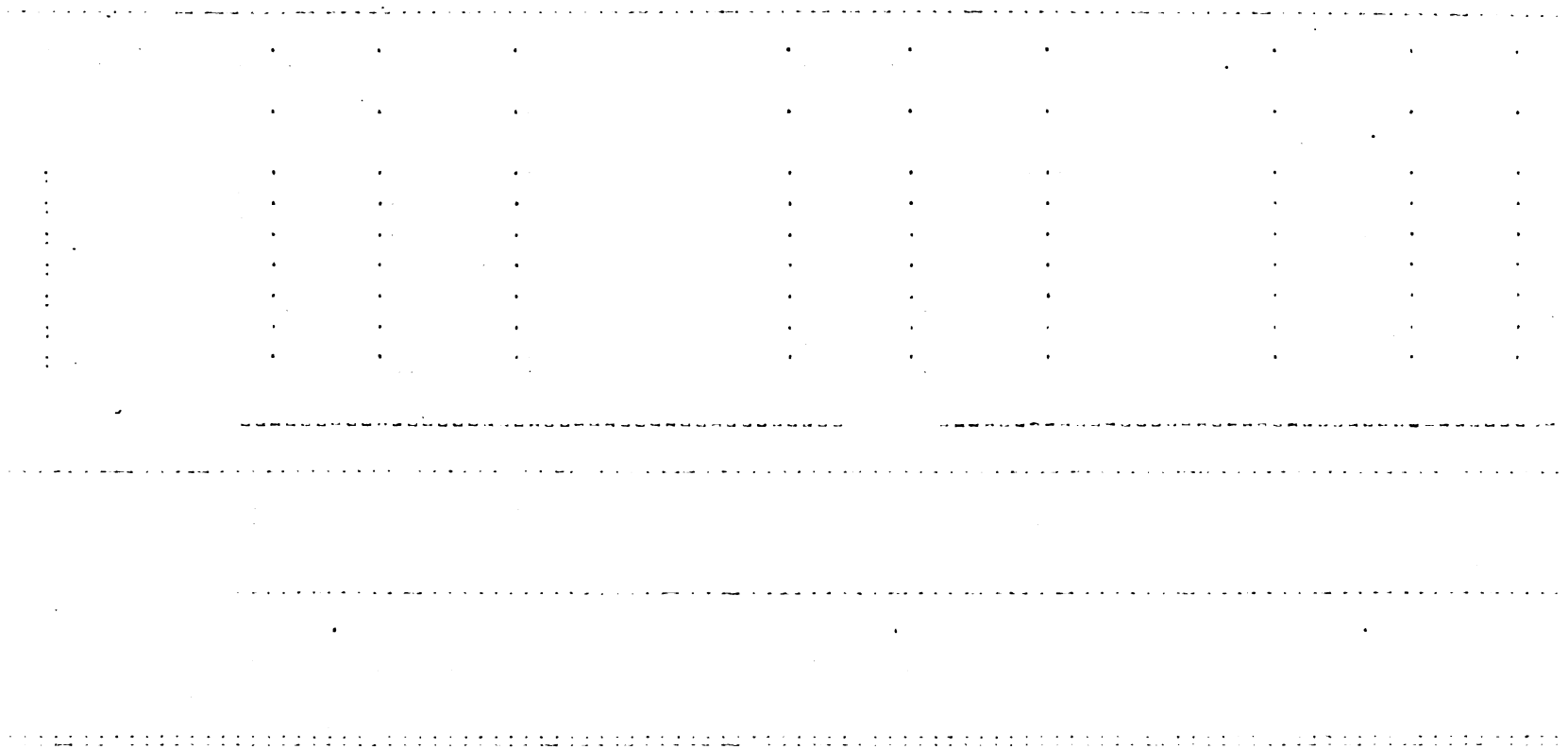


TABLE V

Fatty acid compositions of membrane high-melting triglycerides and the diglycerides and free fatty acids resulting from lipolytic action

Acid	Triglyceride[a]	Diglyceride[b]	Free fatty acids
	Area %		
10:0	1.2	0.9	2.3
12:0	2.6	1.9	4.9
14:0	14.2	19.2	13.3
16:0	46.3	48.4	42.0
18:0	24.9	23.8	22.3
18:1	9.4	8.5	13.1
18:2	1.4	0.3	2.3
Total S	89.3	91.1	84.5
Total U	10.7	8.9	15.3

[a] Average of six samples

[b] Average of three samples

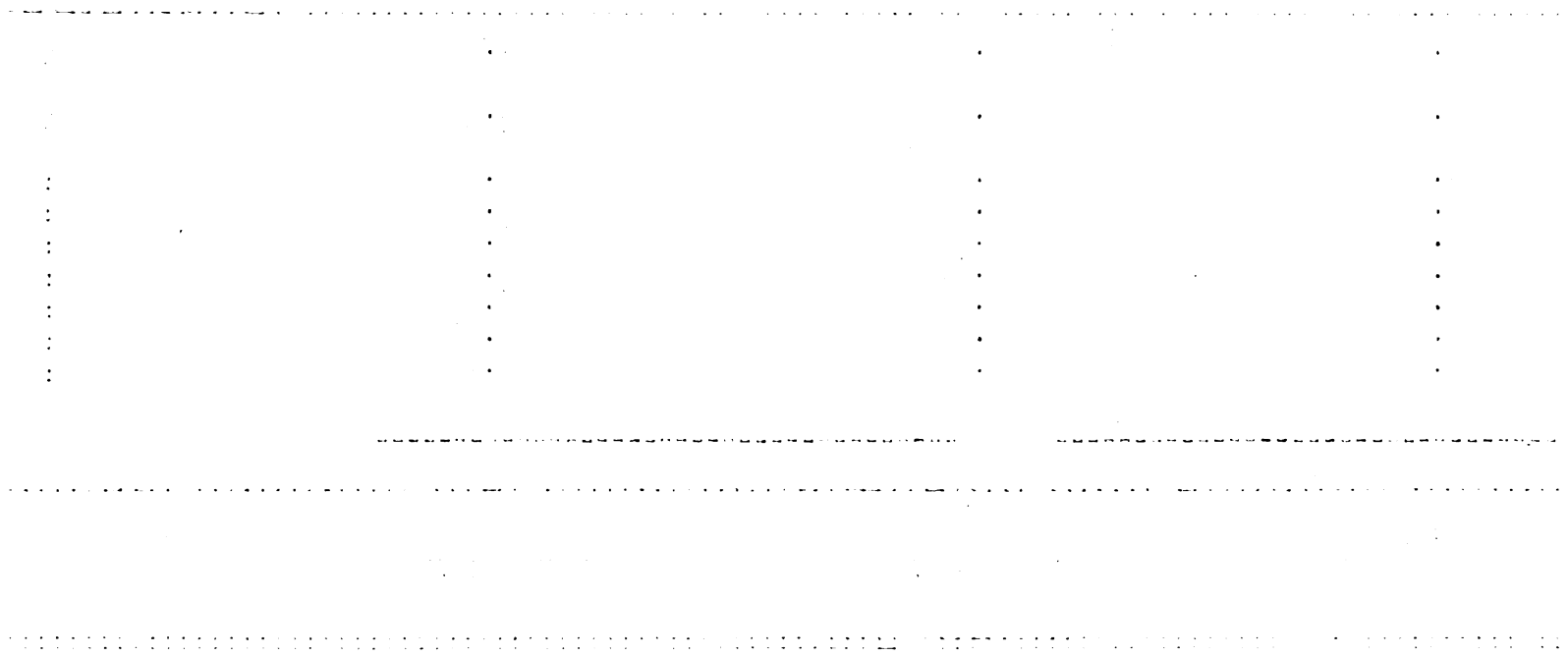


TABLE VI

Triglyceride types and isomeric forms of the fats under study[c]

Fat	Composition: Types (weight %)				Composition: Isomers (weight %)			
	GS_3	GS_2U	GSU_2	GU_3	SUS	SSU	USU	UUS
Butteroil a = 70.2 b = 80.4	34.1	44.8	18.7	2.4	8.3	36.5	9.8	8.9
HMG from butteroil a = 86.5 b = 93.7	64.6	30.7	4.5	0.2	4.3	26.4	2.7	1.8
HMG from membrane a = 89.2 b = 98.4	71.2	26.0	2.8	0.0	1.2	24.8	2.4	0.4

a = % S among the acyl groups in the whole sample as found by analysis

b = % S in the acyl groups in the 2-positions. It is equal to the % S among the acyl groups in the 2-monoglycerides, found by analysis.

[c] Calculated according to the method of VanderWal

TABLE VII

Triglyceride types of the fats under study

Triglyceride types	Butteroil		Butteroil HMG		Membrane HMG	
	Calc.	Random	Calc.	Random	Calc.	Random
S_3	34.1	34.6	64.6	64.7	71.2	71.2
S_2U	44.8	43.9	30.7	30.3	26.0	25.6
SU_2	18.7	18.7	4.5	4.7	2.8	3.1
U_3	2.4	2.6	0.2	0.3	0.0	0.1

DISCUSSION

Isolation of High-Melting Glyceride

The yields of HMG were generally so low that difficulty was encountered in obtaining quantities sufficient for gas chromatographic analysis after digestion and separation of the lipolyzed lipid mixture. The yield of HMG from one gallon of 35% raw cream was between 0.5 and 1.0 g. Increased yields resulted when the membrane was concentrated by lyophilization instead of "salting out" with ammonium sulfate as employed by Thompson et al. (1959). In the salting-out procedure, non-complexed lipid was removed by washing with ethyl ether, however, due to disruption of the lipid-protein complex this non-complexed lipid could not be selectively removed after lyophilization. Because more non-complexed, alcohol soluble lipid was present in those samples that were concentrated by lyophilization, increased amounts of alcohol were required during crystallization.

Thin-layer chromatography indicated that the HMG fraction isolated by precipitation from alcohol contained cholesterol, cholesterol esters and possibly phospholipids (see Figure 2). Inability to quantitatively precipitate phospholipids with acetone would account for their presence in the HMG fraction (Smith and Jack, 1959). Because these contaminants are present in such low concentrations any fatty acids contributed by them would be essentially negligible in the glyceride structure analysis.

Pancreatic Lipase Digestion

The digestion of a fat with pancreatic lipase was allowed to proceed for a maximum of five minutes. Digestion times previously reported range from 15-45 minutes depending on the degree of hydrolysis desired. However,

since early results employing intervals up to 45 minutes were inconsistent, the digestion time was decreased in accord with Jensen (1962). Discordant data from longer periods of digestion could be a result of the migration of fatty acids in the beta position to an alpha carbon or to limited hydrolysis of the fatty acids esterified at the beta position. The specific cause was not established in this study.

The degree of hydrolysis achieved, in a given time, was found to vary with the fats studied. The HMG fractions containing longer, more saturated fatty acids required longer digestion times to achieve the same degree of hydrolysis than did a sample of butteroil or corn oil. Since the HMG is solid at the digestion temperature some question was raised concerning the ability of pancreatic lipase to hydrolyze the triglycerides. However, digestion of a sample of pure tripalmitin (melting point 65.1° C.) was accomplished under the same experimental conditions. Separation of the digestion products on TLC indicated that solid fats as well as liquid fats are hydrolyzed by pancreatic lipase. An explanation for the difference in digestion times lies in the hypothesis of Desnuelle (1961) that pancreatic lipase acts on emulsified esters only. The triglycerides composed of short chain and/or highly unsaturated fatty acids, which are liquid at the temperature employed during digestion, tend to emulsify more readily than the higher melting triglycerides.

Separation of the Digestion Mixture

Since pancreatic lipase is specific for the fatty acids esterified at the one and three positions of a triglyceride, the glyceride structure can be determined by analyzing the triglyceride and the free fatty acids

liberated during lipase hydrolysis. The anion exchange technique described by Hornstein, Alford, Elliott and Crowe (1960) for the recovery of free fatty acids was attempted, but, due to failure to suitably recover the esters of free fatty acids formed directly on the resin, the method was abandoned. McCarthy and Duthie (1962) have recently reported that Amberlite IRA-400 would remove the free fatty acids from a lipid mixture but that quantitative recovery of these acids was virtually impossible.

According to Quinlin and Weiser (1958), glycerides and free fatty acids can be resolved on a column of silica gel. This procedure failed to produce satisfactory separations when used in this laboratory.

Chromatography on Florisil can be used to separate classes of neutral lipids in much the same way as chromatography on silicic acid (Carroll, 1961). Accurate quantitative analysis of a complex lipid mixture by column chromatography, with either adsorbent, is complicated by several factors. One disadvantage is incomplete resolution of components having similar elution characteristics. TLC indicated that the tri-, di- and monoglyceride fractions were not pure in many cases (see Figure 2). Moreover, peak identification by reference to elution volumes of authenic lipids is dependent on exact reproduction of the conditions of analysis. The greatest limitation in achieving reproducible results employing Florisil is in obtaining the proper degree of hydration. This factor has been shown to affect the chromatographic properties of Florisil markedly, changing its adsorption affinity more for some lipids than for others (Carroll, 1961). In addition to column chromatography on Florisil, preparative TLC was employed to separate the digestion

mixture. Difficulty was encountered in esterifying the glyceride fractions scraped from the thin-layer plates that had been made visible by spraying with dichlorofluorescein.

Glyceride Structure

Ideally the fatty acid composition should be expressed as mole percentage. The thermal conductivity detector employed in the gas chromatograph responds on a weight rather than a molar basis. Since the differences between weight percent and mole percent are very small for fatty acids having more than ten carbons and because comparisons of the relative fatty acid compositions of the samples to determine glyceride structure was the objective of this study, the expression of fatty acid data as area percentage was considered adequate. Although errors up to 2% in individual fatty acids are introduced by excluding the minor fatty acids in the calculation of either area percentage or mole percentage, only the chromatographic peak area of the seven major fatty acids were used to compute area percentages.

Butteroil, being readily available, was used to establish the techniques employed in this study. Because no effort was made to recover the short chain fatty acids, the area percentages of the remaining fatty acids were 0-3% higher than those reported by Jensen et al. (1961).

Because of the difficulties experienced in resolving the lipid mixture after lipolysis, fatty acid compositions of monoglycerides and in some cases diglycerides, were not obtained. These data were not crucial, however, as glyceride structure could be determined from

triglyceride and liberated free fatty acid data alone.

Pancreatic lipase analysis on membrane HMG indicate that the beta positions of these glycerides are largely esterified with saturated fatty acids containing 14, 16 or 18 carbon atoms. The averages of the values for the liberated fatty acids and triglyceride composition data permit calculation of the composition of the monoglyceride with the following formula assuming 1,3 random, 2-random distribution: C2=3x (C1,2,3)-2x(C1,3) (VanderWal, 1962). The symbols C1,2,3, C1,3 and C2 in the formula indicate the glycerol carbons to which the corresponding acyl groups are attached.

The monglyceride composition of membrane HMG was calculated to consist of 15% myristic acid, 55% palmitic acid and 30% stearic acid within an error of 2-3%. These results indicate that the beta position of membrane HMG contains only long chain, saturated fatty acids. Obviously, then, any unsaturated fatty acids found in the diglycerides must be esterified at the alpha positions.

The monoglycerides from butteroil HMG was similarly calculated to contain 18% myristic acid, 55% palmitic acid, 19% stearic acid, and 7% oleic acid. The concentrations of myristic acid and stearic acid differ widely from the values obtained by Patton and Keeney (1958), who isolated HMGs from butteroil and membrane by precipitating from acetone (see Table VIII).

The fatty acid compositions of the monoglycerides isolated from hydrolyzed butteroil differed considerably from those calculated by VanderWal's formula. This was not surprising for thin-layer chromatography indicated that many of the di- and monoglyceride fractions were contaminated.

TABLE VIII

A comparison of the fatty acid composition of the fat-globule membrane HMG and HMG isolated from milk fat

Fatty acid	HMG from the fat-globule membrane		HMG from milk fat	
	Area %			
10:0	0.1[a]	1.2[b]	1.7[c]	0.3[d]
12:0	0.9	2.6	3.6	1.4
14:0	11.0	14.2	21.0	12.1
16:0	59.6	46.3	45.7	50.5
18:0	16.5	24.9	14.2	25.5
18:1	5.6	9.4	12.1	4.1[e]
18:2	0.4	1.4	1.4	
Unassigned	5.8	0.0	0.0	5.9

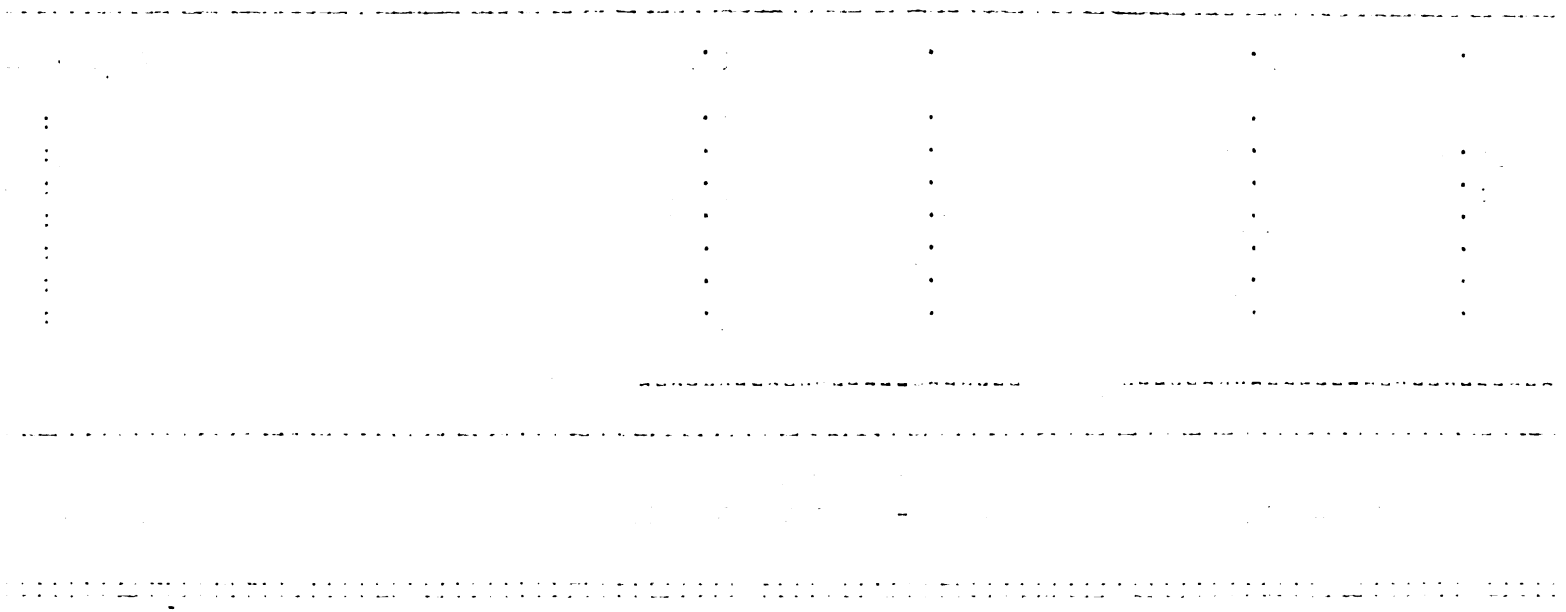
[a]Thompson et al. (1959) obtained identical gas chromatograms for both membrane HMG and butteroil HMG.

[b]Average of six samples.

[c]Average of three samples.

[d]Data of Patton and Keeney (1958).

[e]Total C18 unsaturated.



The fact that butteroil HMG exhibits a lower melting point and a higher unsaturated fatty acid content than membrane HMG indicates that variable triglyceride fractions may be precipitated from alcohol. The variations observed in the degree of unsaturation among butteroil HMG samples appeared to be inversely related to the number of recrystallizations. Therefore, to obtain a pure HMG from butteroil, more than the five crystallizations used for membrane HMG should be employed. The crystallizations should be continued until no glycerides are found in the alcohol supernatant. The difference in the unsaturated fatty acid content of butteroil HMG found in this study and that reported by Patton and Keeney (1958) is due in part to the amount of contamination of the samples analyzed.

Triglyceride Types and Isomeric Forms

The percentage of trisaturated glyceride (GS_3) in butteroil was calculated to be 34.1 by the method of VanderWal (1960), which utilizes the specificity of pancreatic lipase. This value lies within the range reported by Hilditch (1956) of 15-41% GS_3 for butteroil. Eshelman *et al.* (1960) employing the mercaptoacetic acid method obtained values of 27.2 and 28.0. Attempts to reproduce these results by Eshelman's procedure were unsuccessful.

The data in Table VII show clearly that the calculated values for triglyceride types in the fats studied are similar to those predicted by random distribution. It is also obvious that the fatty acyl groups do not assume positions within the molecules completely at random but become segregated in the 2- and 1-3, positions. Determining triglyceride types by classifying the fatty acyl groups only as saturated and unsaturated is not ideal, for it obscures patterns in the placement of the individual

fatty acids. Forty percent of the stearic and palmitic acids occurring in the beta position of membrane HMG is not compatible with the 33% predicted by random distribution. This leads to the observation that while random distribution based on S and U may occur, the individual fatty acids are not randomly distributed in the triglycerides studied.

The results indicate that the membrane HMG contains 71.2% trisaturated glyceride and no triunsaturated (GU_3). Twenty-six percent of the triglycerides are GS_2U but only 1.2% of this fraction is the isomeric form SUS, which contains an unsaturated fatty acid in the beta position. The remaining 2.8% of the membrane high-melting triglycerides are GSU_2 of which 2.4% comprise the USU isomer. The butteroil and the butteroil high-melting glycerides are comparable in isomer form to those of membrane HMG. However, butteroil contains only 34.1% GS_3 while butteroil HMG contains 64.6%.

In butteroil 17.2% of the triglycerides contain an unsaturated fatty acid in the beta position. This figure is reduced to 6.1% in butteroil HMG and 1.6% in membrane HMG based on pancreatic lipase hydrolysis data. Butteroil and the HMG fractions isolated from butteroil and membrane lipid are similar to pig fat, in that the predominant glyceride forms are those which contain a saturated fatty acid in the beta position.

While the isomeric forms calculated for membrane HMG indicate that the beta position of these glycerides contains some unsaturated fatty acid, it appears from monoglyceride calculation that this position in membrane HMG is esterified only with saturated fatty acids.

The significance of the membrane HMG containing beta saturated fatty acids of 14, 16 or 18 carbon atoms is not realized at the present time. Are these HMG molecules selectively adsorbed by the phospholipids or do they, solely on the basis of their physical properties, migrate to the interface where they are complexed with phospholipids?

So little is known concerning lipid-lipid interaction that it is beyond the scope of this study to speculate regarding the nature of the complex, if any, formed between the membrane HMG and the phospholipids associated with the milk fat-globule membrane.

SUMMARY AND CONCLUSIONS

Recently some interest has centered around a high-melting glyceride isolated from the lipid associated with the membrane surrounding the milk fat-globule. The purpose of this study was to determine the structure of this ethanol-insoluble glyceride.

The procedure for the determination of glyceride structure was accomplished in three steps: (1) digestion of the triglyceride with pancreatic lipase, (2) adsorption chromatographic separation of fatty acids and glycerides on Florisil columns, and (3) gas-liquid chromatography of the esters of the fatty acids from the separated fractions.

Structure analysis of the HMG isolated from membrane lipid indicate that the beta position of these glycerides is primarily, if not entirely, occupied by a saturated fatty acid of 14, 16 or 18 carbon atoms.

The results indicate that membrane HMG was composed of 71.2% trisaturated glyceride and no triunsaturated glyceride. Those isomeric forms of GS_2U and GSU_2 which contained a saturated fatty acid in the beta position were found to predominate. The butteroil and butteroil high-melting glycerides, while containing smaller amounts of GS_3, were found to contain the same predominant isomeric forms as the membrane HMG.

Calculation of triglyceride types and isomeric forms led to the conclusion that, while random distribution was indicated on the basis of the distribution of saturated and unsaturated, the individual fatty acids were not randomly distributed in the triglycerides under study.

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