ethylated anionic sulfates, and nonionics consists of the quantitative ion exchange separation of the total nonionic after hydrolysis of the mixture with acid.

The total nonionic will consist of the amount originally present in the sample as free nonionic, and the amount of nonionic derived from the hydrolysis of the anionic sulfate. Subtraction of the amount of free nonionic from the total yields the anionically combined nonionic. This may then be factored to anionic sulfate. If this approach is used, the alkyl aryl sulfonate may be determined by cationic titration of a suitable aliquot after the hydrolysis step. This method is not applicable to mixtures containing alkanolamide additives or to nonionics that are hydrolyzed under conditions of the test.

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REFERENCES

1. Spangler, W. G., JAOCS 34, 191 (1957).
2. Am. Soc. Testing Materials, "Bibliographical Abstracts of Methods for Analysis of Synthetic Detergents," Special Technical Publications Nos. 150, 150-B and 150-C.
3. Rosen, M. J., and H. A. Goldsmith, "Systematic Analysis of Surfac-Active Agents," Interscience Publishers, Inc., New York (1960).
4. Am. Soc. Testing Materials, "ASTM Standards on Soaps and Other Detergents," Ninth Ed., Philadelphia (1960), p. 139.
5. AOCS, "Official and Tentative Methods," Method Db 2-48 (1961).
6. Weeks, L. E., and J. T. Lewis, JAOCS 37, 138 (1960).
7. Ross, L. U., and E. W. Blank, *Ibid.* 34, 70 (1957).
8. Kortland, C., and H. F. Dammers, *Ibid.* 32, 58 (1955).
9. Smith, W. B. Analyst 84, 77 (1959).
10. Bergeron, J., R. Derenemesnil, J. Ripert, and G. Monier, Bull, mens, inform. ITERG (Inst. tech, etudes recherches corps gras) 4, 118 (1950); C. A. 44, 6172 (1950).
11. Jenkins, J. W., and K. O. Kellenbach, Anal. Chem. 31, 1056 (1959).
12. Delsemme, A. H., 1st World Congress on Surface Active Agents, Vol. 1, p 192 (1954).
13. Sadtler, S. P., ASTM Bull. 190, 51 (1953).
14. Reid, V. W., T. Alston, and B. W. Young, Analyst 80, 682 (1955).
15. Palit, S. R., Oil and Soap 23, 58 (1946).
16. House, R., and J. L. Darragh, Anal. Chem. 26, 1492 (1954).
17. Kelley, R M., E. W. Blank, W. E. Thompson, and R. Fine, ASTM Bull. 237, 70 (1959).
18. Blank, E. W., Soap Chem. Specialties 34, 41 (1958).
19. Cf. Ref. 4, p. 194.
20. Strouts, C. R. N., J. H. Gilfillan, and H. N. Wilson, Editors, "Analytical Chemistry," Vol. 2, Oxford University Press, London, 1955, p. 694.
21. Cf. Ref. 4, p. 153.
22. Sadtler, S. P., Spectra of Surface Active Agents, Sadtler Research Laboratories, Philadelphia.
23. Colthup, N. B., J. Opt. Soc. Am. 40, 397 (1950).
24. Ginn, M. E., and C. L. Church, Anal. Chem. 31, 551 (1959).

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Structure of High Melting Glycerides from the Milk Fat-Globule Membrane[1]

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Abstract

The method for determining the position of a fatty acid in triglycerides with pancreatic lipase has been applied to high-melting glycerides from the milk fat-globule membrane.

Definition of location of individual fatty acids within the membrane high-melting triglycerides 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 fatty acids, the individual fatty acids were not randomly distributed in the triglycerides under study.

Introduction

PALMER AND Weise (1) were the first to report the isolation of a high-melting glyceride (HMG) from the milk fat-globule membrane by precipitation from ethanol at room temperature. In 1945, Jenness and Palmer (2) isolated and characterized HMG fractions of similar properties from butterfat, washed-cream buttermilk extracts, and washed-cream serum extracts. More recently Patton and Keeney (3) and Thompson, Brunner, and Stine (4) have published the gas chromatographic analysis of the fatty acid composition of HMG fractions precipitated from acetone and ethanol respectively. This study was undertaken to determine the structure of ethanol-insoluble, high-melting glycerides isolated from membrane lipid, and to compare this fraction with the high-melting glycerides from butteroil.

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Experimental

Figure 1 represents the procedure for isolating HMG from the milk fat-globule membrane. Washed cream was churned and the butterfat was separated from the buttermilk in a laboratory separator. The membrane containing buttermilk was lyophilized and the lipids were extracted with ethyl ether and 95% ethanol. Phospholipids were precipitated with acetone and the HMG fraction was purified by crystallizing from 95% ethanol.

Since the fatty acyl groups esterified at the one and three position of a triglyceride are reported to be selectively removed by pancreatic lipase, this enzyme provides a convenient approach to the determination of triglyceride structure (5,6). The procedure follows: 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.

The digestion mixture was 0.5 g triglyceride, 20 ml distilled water, 0.5 ml 45% aqueous solution $CaCl_2$, 0.2 ml 1% aqueous solution of bile salts, and 100 mg pancreatic lipase (Mann Research Laboratories, pork pancreas-crude). Digestion was at 40C with continuous agitation. The pH was held at 8 by periodic additions of 0.1 *N* NaOH, and digestion proceeded 5 min. After the hydrolysis period, pH was reduced

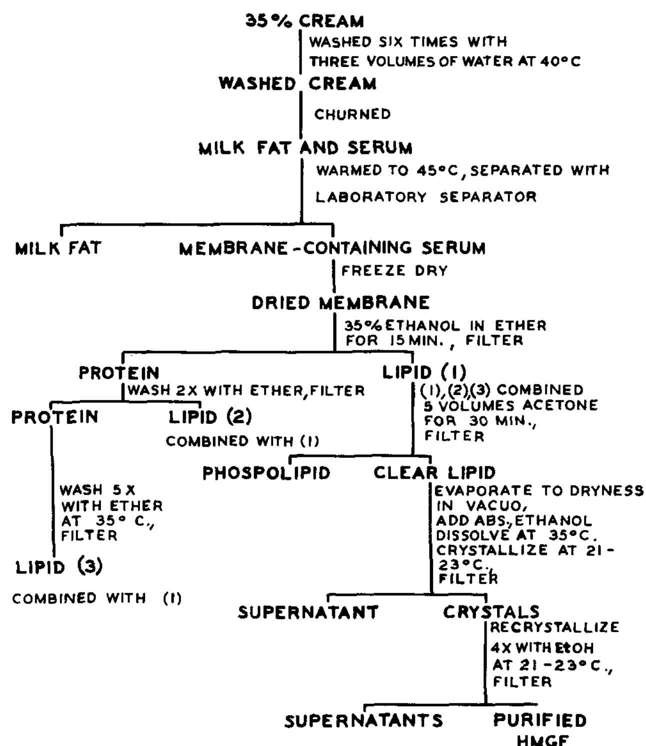

FIG. 1. Schematic representation of procedure for isolation of HMG from milk fat-globule membrane.

below 3.0 with 6*N* HCl. The lipids were then recovered by extraction with petroleum ether. The P.E. solution was washed with water, dried with sodium sulfate, and the solvent removed under vacuum. Neutral glycerides and fatty acids liberated during digestion were separated by adsorption column chromatography on Florisil according to Carroll (7). The column eluate was collected in 25 ml portions, the solvent removed, and the purity of each fraction ascertained by TLC on silicic acid (Fig. 2). Methyl esters of the neutral glyceride fatty acids were prepared by the base-catalyzed interesterification method of Smith and Jack (8). The free fatty acids were esterified by refluxing in methanol with HCl or H_2SO_4. Aerograph Model A-90-C Gas Chromatograph was used with Leeds and Northrup Type G Speedomax recorder. The column was packed with 20% diethylene glycol succinate on acid-washed firebrick, and was operated at 180C. Helium was the carrier gas, with outflow rate 50–100 cc/min. Component analysis was done by measuring the areas of the peaks with a planimeter. The area percentage for the major fatty acids, based on the total area of the major fatty acids, was computed. Unidentifiable area and minor fatty acid area were not included in this calculation.

Results

The values for the fatty acid analyses of butteroil triglycerides and the diglycerides, monoglycerides, and free fatty acids resulting from lipolytic action on butteroil 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

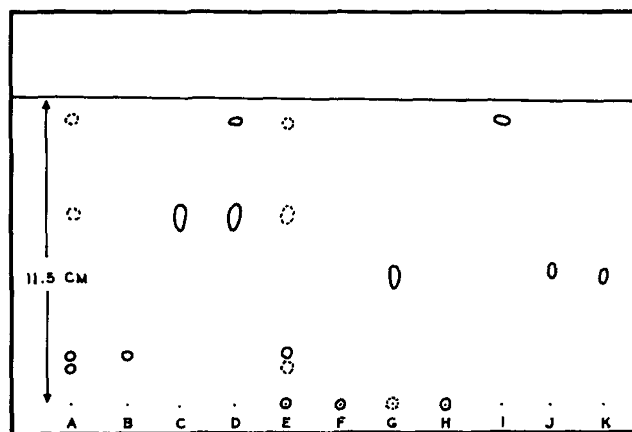

FIG. 2. Thin-layer adsorption chromatography of lipid classes on Silica Gel. G. Solvent: petroleum ether-ethyl ether-acetic acid, 90/10/1, v/v/v. Development time: 40 min. Indicator: dichlorofluorescein. Quantity: ca. 20 λ each. a) diglyceride from Florisil column, b) cholesterol, c) tripalmitin, d)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

substantiated by analysis of the free fatty acids which indicated higher than random concentrations of 10:0 and 18:1 esterified at the alpha position of butteroil.

Data in Table I agree well with those of McCarthy, Patton, and Evans, (9,10), and Jensen, Sampugna, and Gander (11).

The results from the lipolysis of HMG isolated from butteroil are 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.

Results for HMG from the fat-globule membrane are in Table III. In most respects data were similar to those exhibited by HMG from butteroil, indicating a higher than random concentration of 16:0 and 18:0 in the β position of this triglyceride fraction. In contrast to 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 dissimilarities in percentages of the saturated fatty acids myristic and stearic: see Tables II and III.

The proportions of the triglyceride types and isomeric forms were computed for the fats studied by the method of Vander Wal (12), and the results show in Table IV.

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
	Area %	Area %	Area %	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	34.7	38.4	39.5	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 in the fatty acid.
[b] Avg of four samples.
[c] Avg of three samples.

TABLE II

Fatty Acid Compositions of Butteroil High-Melting Triglycerides and the Free Fatty Acids Resulting from Lipolytic Action.[a]

Acid	Tri-glyceride	Free fatty acids
	Area %	Area %
10:0	1.7	2.1
12:0	3.6	5.5
14:0	21.0	22.0
16:0	45.8	41.7
18:0	14.2	11.7
18:1	12.2	14.8
18:2	1.4	2.2
Total S	86.5	82.9
Total U	13.5	17.0

[a] Avg of three samples.

Discussion

The digestion of a fat with pancreatic lipase was allowed to proceed for a maximum of 5 min. Digestion times previously reported range from 15–45 min, depending on the degree of hydrolysis desired. However, since early results employing intervals up to 45 min were inconsistent, digestion time was decreased in accordance with Jensen (13). Discordant data from longer periods of digestion could result from the migration of fatty acids in the β position to an α carbon or to limited hydrolysis of the fatty acids esterified at the β position. The specific cause was not established in this study, although migration would tend to be ruled out according to the results of Mattson and Volpenhein (14). It served, however, to raise questions regarding the specificity and selectivity of the lipolytic enzyme or enzymes in the preparation used for this study.

Since high-melting glycerides are solid at the temperature employed for digestion, it was necessary to ascertain the ability of pancreatic lipase to hydrolyze a solid fat. A sample of tripalmitin, mp 65.1C, was digested under the same conditions. Separation of the digestion products by TLC indicated that solid fats as well as liquid fats are hydrolyzed by the enzyme.

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). The symbols C1,2,3, C1,3 and C2 in the formula indicate the glycerol carbons to which the corresponding acyl groups are attached. This calculation was suggested by Vander Wal (15).

The monoglyceride composition of membrane HMG excluding unassigned area was calculated to consist of 15% myristic, 55% palmitic, and 30% stearic acid.

The monoglyceride from butteroil HMG was similarly calculated to contain 18% myristic, 55% palmitic, 19% stearic, and 7% oleic acid. The composition of butteroil HMG fractions reported here differ from that reported by Thompson et al. Butteroil HMG was found to contain a higher concentration of myristic acid than membrane HMG isolated from the same preparation.

Data in Table IV show that calculated values for triglyceride types in the fats studied are similar to those predicted by random distribution. It can also be seen 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 acids only as saturated and/or unsaturated is not ideal, for it obscures patterns in the positioning of the individual fatty acids. A higher than random percentage of stearic and palmitic acids were found to occur in the β position of the glycerides studied. This indicated that while random distribution based on S and U apparently occurs, the individual fatty acids were not randomly distributed.

Membrane HMG was found to contain 71.2% trisaturated glyceride and essentially no triunsaturated glyceride. Of the triglycerides 26% were GS_2U but only 1.2% of this fraction was the isomeric form SUS. The remaining 2.8% of the membrane high-melting triglycerides were GSU_2, 86% of which were the USU isomer. The butteroil and butteroil high-melting glycerides were comparable in isomer form to those of membrane HMG. However, butteroil contained only 34.1% GS_3 while butteroil HMG contained 64.5%.

Butteroil and the HMG fractions isolated from butteroil and membrane lipid appeared to be similar to pig fat, in that the predominant glyceride forms were those which contained a saturated fatty acid in the β position.

TABLE III

Fatty Acid Composition of Membrane High-Melting Triglycerides and the Diglycerides and Free Fatty Acids Resulting from Lipolytic Action.

Acid	Tri-glyceride [a]	Di-glyceride [b]	Free fatty acids
	Area %	Area %	Area %
10:0	1.2	0.9	2.3
12:0	2.6	1.9	4.9
14:0	14.2	18.1	13.3
16:0	46.3	47.0	42.0
18:0	24.9	23.3	22.2
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] Avg of six samples.
[b] Avg of three samples.

TABLE IV

Triglyceride Types and Isomeric Forms of the Fats Under Study.[c]

Fat	Composition: Types (wt %)				Composition: Isomers (wt %)			
	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 by the method of Vander Wal (12).

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REFERENCES

1. Palmer, L. S., and H. F. Weise, J. Dairy Sci., *41*, 1288 (1933).
2. Jenness, R., and L. S. Palmer, *Ibid.*, *28*, 653 (1945).
3. Patton, S., and P. G. Keeney, *Ibid.*, *41*, 128 (1958).
4. Thompson, M. P., J. R. Brunner, and C. M. Stine, *Ibid.*, *42*, 1651 (1959).
5. Mattson, F. H., and L. W. Beck, J. Biol. Chem., *214*, 115 (1955).
6. Mattson, F. H., and L. W. Beck, *Ibid.*, *219*, 735 (1956).
7. Carroll, K. K., J. Lipid Research, *2*, 135 (1961).
8. Smith, L. M., and E. L. Jack, J. Dairy Sci., *37*, 380 (1954).
9. McCarthy, R. D., S. Patton, and Laura Evans, *Ibid.*, *43*, 1196 (1960).
10. Patton, S., Laura Evans, and R. D. McCarthy, *Ibid.*, *43*, 95 (1960).
11. Jensen, R. G., J. Sampugna, and G. W. Gander, *Ibid.*, *44*, 1983 (1961).
12. Vander Wal, R. J., JAOCS, *37*, 18 (1960).
13. Jensen, R. G., Private communication, (1962).
14. Mattson, F. H., and R. A. Volpenhein, J. Lipid Res., *2*, 58 (1961).
15. Vander Wal, R. J., Unpublished data, (1962).

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