and 3 positions in the original fats as 29.2% saturated and 70.8% unsaturated. After oxidation the total fatty acid composition was 43.4% saturated and 56.5% dicarboxylic. The composition in the 1 and 3 positions was 28.1% saturated and 71.9% dicarboxylic. This oxidized material was split into two fractions on the liquid-liquid partition column. The analysis of the first fraction was: total composition, 73.1% saturated acids and 26.9% dicarboxylic acids; 1 and 3 positions, 60.0% saturated acids and 40.0% dicarboxylic acids. The analysis of the second fraction was: total composition, 27.6% saturated acids and 72.4% dicarboxylic acids; 1 and 3 positions, 12.8% saturated acids and 87.2% dicarboxylic acids.

The relative amounts of the two fractions were calculated from a material balance of the saturated acids. Let X be the mole fraction of the total glycerides in the first fraction. Then

$$73.1\,X + 27.6\,(1 - X) = 43.4$$
$$X = 0.347$$
$$1 - X = 0.653$$

Fraction 1 contained only $S_3$ and $S_2U$ glyceride types so that each dicarboxylic acid present (equivalent to the original unsaturated acids) must be associated with two saturated acids as $S_2U$ and the mole percentage of this material in fraction 1 was $3 \times 26.9 = 80.7\%$. The remaining 19.3% was $S_3$. Of the two possible $S_2U$ isomers, SUS and SSU, only the unsymmetrical isomer would give rise to dicarboxylic acids on lipase hydrolysis. There is one saturated acid for every dicarboxylic acid in the 1 and 3 position of this isomer, and the amount was $2 \times 40.0 = 80.0\%$. Since the total $S_2U$ was 80.7%, there was only 0.7% of the SUS isomer. Multiplying the amounts of each of the three glyceride types by the mole fraction of material in Fraction 1 (0.347) gave the amounts in the total sample as SSS 6.7%, SSU 27.8%, and SUS 0.2%.

Similarly for Fraction 2, which contained SUU, USU, and UUU, the total amout of $SU_2 = 3 \times 27.6 = 82.8\%$. By difference the UUU = 17.2%. The amount of $SUU = 2 \times 12.8 = 25.6\%$ and USU, $82.8 - 25.6 = 57.2\%$. Multiplying by the mole fraction of material in Fraction 2 gives the amounts in the total sample as SUU 16.7%, USU 37.4%, and UUU 11.2%.

Rounding these figures off to the nearest percentage gives the final calculated composition as: SSS 7%, SSU 28%, SUS 0%, SUU 17%, USU 37%, and UUU 11%. The composition expected on lipase hydrolysis of a fat of this composition would be 29% saturated and 71% unsaturated acids. The composition found for the original fat was 29.2% saturated and 70.8% unsaturated acids.

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# The Structural Components of Milk Triglycerides[1]

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Pancreatic lipase hydrolysis and gas chromatographic analysis of two samples of butter fat show that the individual acyl groups are not dispersed at random among all the glyceryl carbons. When considered only as saturated or unsaturated, and not as individuals, they appear to be distributed intermolecularly at random, or nearly so, but tend to assume specific positions intramolecularly.

THE LITERATURE contains many accounts of component fatty acid analysis of milk fat triglycerides, and some record of work on the component triglycerides. Much of the work has been referred to by Hilditch (1). Recently Bhalerao, Johnson, and Kummerow (2) have reported results of the triglyceride type of analysis[2] of a sample of butter fat by fractional crystallization. The percentages found were approximately those that would be expected if the S and U were distributed at random throughout the molecules of the fat.

More recently Patton, Evans, and McCarthy (3) have published the results of pancreatic lipase hydrolysis and gas chromatographic analysis of a sample of summer milk fat.[3]

In the present paper the results of similar analyses of milk fat will be reported. The percentages of the triglyceride types and isomeric forms in terms of S and U, calculated by the method of Vander Wal (4), will also be presented and compared with the values calculated in the same way from the data of Patton *et al.*

## Experimental

*Samples.* Sample 1 was commercially produced butter oil made by conventional processes and further clarified by filtration. Sample 2 was obtained from high quality commercial butter by evaporation of the water phase under reduced pressure, extraction of the residue with Skellysolve F, drying with sodium

[1] Presented at the 51st annual meeting, American Oil Chemists' Society, Dallas, Tex., April 4–6, 1960.

[2] The triglyceride types are $S_3$, $S_2U$, $SU_2$, and $U_3$. S stands for saturated acids or acyl groups, and U stands for unsaturated acids or acyl groups.

[3] In a personal communication Dr. Patton has stated that the initial, rough separation of the fat from the aqueous portion was as butter fat.

sulfate, filtration, and removal of the solvent by evaporation. The butter had been packaged in Minnesota in February 1960 and was therefore "winter" butter.

*Pancreatic Lipase Hydrolysis.* The general procedure for pancreatic lipase hydrolysis used in this laboratory and employed in the present experiments is as follows. One gram of the sample, 1.5 g. NaCl, and 35 ml. of 1% Elvanol [4] solution are heated with stirring to 45°C. The pH is adjusted to 7.0–7.5 with 1N NaOH solution. The finely divided pork pancreatic lipase [5] is then added. Ordinarily 100 mg. are employed for low-melting fats such as butter and coconut oil and 200 mg. for higher melting ones such as lard. Stirring is continued at 45°C., and the pH is maintained at 7.2–8 by periodic addition of 1N NaOH solution. When 20 to 30% of hydrolysis has occurred as indicated by the quantity of NaOH added, the pH is reduced to below 3.0 with 6N HCl solution. The digestion process takes between 15 and 45 min. The mixture is then extracted continuously for 90 min. with 40 ml. of Skellysolve F. The solvent is evaporated and the residue is treated with diazomethane.[6] After removal of the solvent the methyl esters are stripped from the unreacted glycerides at 240°C. and 13 mm. Hg in a stream of $CO_2$ and are collected in a dry ice trap. The sample is then ready for analysis.

### Preparation of the Methyl Esters of the Whole Fat

One hundred milligrams of the fat were heated with 6–8 ml. of anhydrous methanol containing an excess of KOH (one pea-size pellet is sufficient) until saponification was complete. Temperature was raised to boiling point, and solution stirred until its volume was decreased to about 2–4 ml. About 1 ml. of $H_2O$ was added, and heating and stirring were continued until the methanol was evaporated. The residue was diluted with about 4 ml. of $H_2O$ and the pH reduced to 3.0, or below, with 6N HCl. Stirring and heating were continued until the upper layer was a liquid, after which it was extracted with four to six 4-ml. portions of Skellysolve F. The extracts were combined, and the solvent was removed on a steam bath.

The residual fatty acids were treated with diazomethane as previously described, and the resultant methyl esters were analyzed by gas chromatography.

### Gas-Liquid Chromatographic Analysis of the Methyl Esters

The 6-ft. column employed in the analyses was packed with 60–100 mesh, treated Chromosorb W coated with 35% diethylene glycol succinate polyester. Helium flow and temperature were adjusted so that methyl palmitate was eluted in about 15 min. The thermal conductivity detector system was maintained at 240°C. Peak areas were determined by triangulation and identified, for the most part, by comparison with known results. Other peaks were tentatively identified by their position on the carbon chain length-elution time curve of an n-saturated homologous series plotted on semi-log paper.

### Results and Discussion

Results of the application of these procedures to the two samples of butter fat are shown in Table I.

The data in Table I show that the various individual fatty acyl groups in commercial butter fat are not distributed at random among the glyceryl carbons and that there is some quantitative variation between samples. Greater-than-random percentages of $C_{14}$, $C_{16}$, and $C_{16}'$ acids are esterified at the 2-position of the glycerol moiety, and less-than-random proportions of $C_{18}$ and $C_{18}'$ fatty acids are esterified at the same position. These results agree with those of Patton *et al.*

TABLE I

Component Acids (% Wt.) in Butter Fat

| | Sample 1 | | | Sample 2 | | |
|---|---|---|---|---|---|---|
| | Esterified at [a] | | | Esterified at | | |
| | C 1,2,3 | C 1,3 | C 2 | C 1,2,3 | C 1,3 | C 2 |
| $C_3$ [b] | ..... | ..... | ..... | T | T | ..... |
| $C_4$ | ..... | ..... | ..... | 0.61 | T | 1.83 |
| $C_{4.5}$ [c] | ..... | ..... | ..... | T | T | ..... |
| $C_5$ [b] | ..... | ..... | ..... | T | T | ..... |
| $C_6$ | 0.95 | 1.07 | 0.71 | 1.88 | 1.52 | 2.60 |
| $C_7$ [b] | ..... | ..... | ..... | T | T | ..... |
| $C_8$ | 1.35 | 1.72 | 0.61 | 1.28 | 1.57 | 0.70 |
| $C_9$ [b] | ..... | ..... | ..... | T | T | ..... |
| $C_{10}$ | 2.98 | 3.18 | 2.58 | 2.66 | 2.65 | 2.68 |
| $C_{11}$ [b] | 0.41 | 0.44 | 0.35 | 0.24 | 0.45 | −0.18 |
| $C_{12}$ | 3.26 | 3.56 | 2.66 | 2.92 | 3.04 | 2.68 |
| $C_{13}$ | 0.21 | 0.21 | 0.21 | 0.20 | 0.12 | 0.36 |
| $C_{13.5}$ [c] | ..... | ..... | ..... | 0.17 | T | 0.51 |
| $C_{14}$ | 10.26 | 8.48 | 13.82 | 10.18 | 8.11 | 14.32 |
| $C_{14}'$ [b,d] | 3.41 | 2.58 | 5.07 | 0.24 | 0.11 | 0.50 |
| $C_{14.8}$ [c] | | | | 1.38 | 1.38 | 1.38 |
| $C_{15}$ | | | | 1.09 | 1.07 | 1.13 |
| $C_{14}''$ [b] | ..... | ..... | ..... | 0.45 | 0.25 | 0.85 |
| $C_{16}$ | 25.77 | 24.57 | 28.17 | 31.19 | 27.02 | 39.53 |
| $C_{16}'$ | 2.61 | 2.00 | 3.83 | 3.53 | 2.75 | 5.09 |
| $C_{17}$ | 0.83 | 0.87 | 0.75 | 0.99 | 1.25 | 0.47 |
| $C_{16}''$ [b] | ..... | ..... | ..... | 0.41 | 0.58 | 0.07 |
| $C_{18}$ | 13.15 | 14.91 | 9.63 | 12.47 | 14.81 | 7.79 |
| $C_{18}'$ | 29.28 | 31.86 | 24.12 | 25.17 | 30.07 | 15.37 |
| $C_{19}$ | ..... | ..... | ..... | 0.28 | 0.40 | 0.04 |
| $C_{18}''$ | 2.52 | 2.12 | 3.32 | 1.56 | 1.62 | 1.44 |
| $C_{20}$ | ..... | ..... | ..... | 0.22 | 0.34 | −0.02 |
| $C_{18}'''$ | 2.32 | 2.58 | 1.80 | 0.96 | 0.90 | 1.08 |
| Total S | 62.58 | 61.59 | 64.56 | 67.76 | 63.73 | 75.82 |
| Total U | 36.73 | 38.56 | 33.07 | 32.32 | 36.28 | 24.40 |

[a] Values under headings C 1,2,3, etc., are % (wt.) of the component acid in the acids esterified at the so designated glyceryl carbon atoms.
[b] Peaks tentatively identified by position on carbon chain length–elution time curve.
[c] Apparent carbon chain length. May be branched chain acids.
[d] The mark ' indicates one double bond. Thus $C_{18}'$ stands for monoene, and $C_{18}''$ stands for diene.

A greater net percentage of S is esterified at the 2-position than at the 1- and 3-positions, and this result is also in agreement with that of Patton, who pointed out that in this respect butter fat is similar to lard.

The proportions of the triglyceride types $S_3$, $S_2U$, $SU_2$, and $U_3$, and the isomeric forms of $S_2U$ and $SU_2$ calculated by the method of Vander Wal (4) are presented in Table II. The same procedure was applied to the data by Patton *et al.*, and the results are included in the table. Separate calculations were made from the data for "diglycerides," which are equivalent to the column headed C 1,3 in Table I, and those headed "monoglycerides," which correspond to the column headed C 2 in Table I.

The percentages of the isomeric forms SUS, SSU, USU, and UUS shown in Table II do not agree completely with the corresponding values for random dis-

[4] A partially acetylated polyvinyl alcohol produced by E. I. du Pont de Nemours and Company.

[5] Lipase powder (pork pancreas, crude) purchased from Mann Research Laboratories, New York, N. Y., has been found satisfactory without treatment.

[6] The diazomethane is prepared by adding, dropwise, a solution of 200–400 mg. of N-nitroso-N-methyl-paratoluenesulfonamide in 4 ml. of a mixture of equal parts of ethyl ether and 95% ethanol, directly onto 400 mg. of powdered sodium methoxide in a small distilling flask maintained at 50°C. The diazomethane so generated is distilled, along with the ether vapors, onto the sample to be methylated and, some of it, subsequently into 4 ml. of ether. The sample is considered methylated when both its ether solution and the ether next in the train become strongly yellow from dissolved diazomethane. The solvent and excess diazomethane are then removed by distillation or evaporation. Diazomethane is highly toxic, and all operations must be carried out in an efficient hood. It is also spontaneously explosive, and precautions should be taken against injury.

TABLE II

Triglyceride Types and Isomers (% Wt.) in Butter Fat and Milk Fat

| | Butter fat | | | | Milk fat[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | Sample 1 | | Sample 2 | | Calculated from data on | | Average | Random |
| | Calc. | Random | Calc. | Random | Diglycerides | Monoglycerides | | |
| SSS | 24.5 | 24.5 | 30.9 | 31.2 | 28.1 | 28.0 | 28.1 | 28.2 |
| SUS | 13.4 | 14.7 | 9.9 | 14.8 | 17.3 | 10.1 | 13.7 | 14.8 |
| SSU | 30.6 | 29.4 | 35.0 | 29.6 | 27.3 | 34.7 | 31.0 | 29.6 |
| USU | 9.5 | 8.8 | 9.9 | 7.0 | 6.6 | 10.8 | 8.7 | 7.8 |
| UUS | 16.8 | 17.6 | 11.2 | 14.1 | 16.7 | 12.5 | 14.6 | 15.6 |
| UUU | 5.2 | 5.2 | 3.1 | 3.3 | 4.0 | 3.9 | 4.0 | 4.1 |
| $S_3$ | 24.5 | 24.5 | 30.9 | 31.2 | 28.1 | 28.0 | 28.1 | 28.2 |
| $S_2U$ | 44.0 | 44.0 | 44.9 | 44.4 | 44.6 | 44.8 | 44.7 | 44.4 |
| $SU_2$ | 26.3 | 26.3 | 21.1 | 21.1 | 23.3 | 23.3 | 23.3 | 23.3 |
| $U_3$ | 5.2 | 5.2 | 3.1 | 3.3 | 4.0 | 3.9 | 4.0 | 4.1 |

[a] Calculated from data by Patton *et al.* (3). The initial, rough separation of the fat from the aqueous phase was as butter fat. The samples are therefore comparable.

tribution. Those for Sample 1 are closer to the random pattern than those for Sample 2.

The values for the triglyceride types in the lower portion of Table II are all close indeed to those which would be found if distribution of S and U were completely at random. Those of Bhalerao and co-workers, which are not included in the table, are not quite as close to the random pattern but not far distant.

The data in Table II show clearly that in butter fat the fatty acyl groups, classified only as saturated (S) and unsaturated (U), have been brought together in groups of three at random, or nearly so; these triplets constitute triglycerides. It is equally obvious from the data in the first part of Table II that they do not assume positions within the molecules completely at random but to some degree become segregated in the 2-, and 1-, 3-positions. In this they behave like the individual fatty acids comprising each group, as recorded in Table I.

Butter fat is, then, another of the group of fats in which S and 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 operating freely or with some restriction. Kartha's mathematical formulae (4,5,6) comprise one method of arriving at this end. In butter fat this circumstance persists in spite of considerable variation in the fatty acid composition and in the distribution of the individual fatty acyl groups.

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# Analysis of the Glyceride Structure of Cocoa Butter by Thermal Gradient Crystallization[1]

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Cocoa butter was separated into 43 fractions by crystallization in a thermal gradient. Similar fractions were pooled and converted into methyl esters which were analyzed by gas-liquid chromatography. The amount of cocoa butter separated into a pure glyceride type was 85%. No significant difference was found in the ratio of palmitic to stearic acid in the $GSU_2$ and $GS_2U$. In the $GS_2U$, ternary and binary eutectic mixtures are predicted by the ideal solution theory. When the eutectics are taken into consideration, the individual glyceride composition of cocoa butter agrees well with the composition predicted by restricted random distribution. To test the ability of thermal gradient crystallization to separate $GU_3$ and $GS_2U$ a sample of cocoa butter plus 10% triolein was analyzed. The apparent composition of the cocoa butter and triolein indicated that the $GU_3$ and the $GS_2U$ separation was incomplete.

CONSIDERABLE WORK has been done on the glyceride structure of cocoa butter because of its valuable physical properties. Hilditch and Stainsby (1) separated cocoa butter into three fractions by crystallization and attempted to deduce its glyceride structure from the fatty acid composition of the fractions. Meara (2) separated cocoa butter into 11 fractions by "exhaustive" crystallization and computed the glyceride structure in a like manner. In making these calculations, it was necessary to assume that each fraction contained only two glycerides. While the present work was in progress, Scholfield and Dutton (3) published a paper describing the analysis of cocoa butter by using countercurrent dis-

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