Floyd E. Kurtz

The Lipids of Milk: Composition and Properties

INTRODUCTION

Milkfat consists chiefly of triglycerides of fatty acids. It also contains varying quantities of other compounds as indicated in Table 4.1. Some additional materials are included in the text. Others are undoubtedly present but still unidentified. The relative proportions of these compounds in milkfat are affected by such factors in its production as the type and amount of feed, stage of lactation, and species of animal or breed of cow. Constituents not normally present may be incorporated

Table 4.1

COMPOSITION OF BOVINE MILK LIPIDS

Class of Lipid	% Total Milk Lipids
Triglycerides of fatty acids	95–96
Diglycerides	1.26–1.59
Monoglycerides	0.016–0.038
Keto acid glycerides (total)	0.85–1.28
Ketonogenic glycerides	0.03–0.13
Hydroxy acid glycerides (total)	0.60–0.78
Lactonogenic glycerides	0.06
Neutral glyceryl ethers	0.016–0.020
Neutral plasmalogens	0.04
Free fatty acids	0.10–0.44
Phospholipids (total)	0.80–1.00
Sphingolipids (less sphingomyelin)	0.06
Sterols	0.22–0.41
Squalene	0.007
Carotenoids	0.0007–0.0009
Vitamin A [a]	0.0006–0.0009
Vitamin D	0.00000085–0.0000021
Vitamin E	0.0024
Vitamin K	0.0001

[a] Based on the free alcohol.

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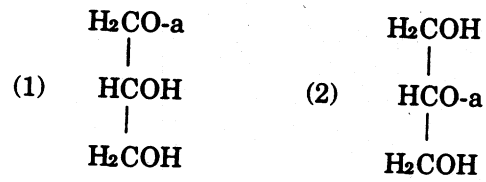
into milkfat under unusual feeding conditions. The proportions of some of these components may vary widely with dairy processing. Butterfat prepared from churned cream, for example, may be devoid of detectable phospholipids. It is, therefore, incorrect to use the terms "milkfat" and "butterfat" interchangeably.

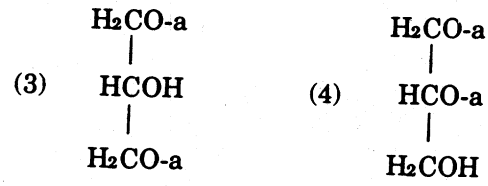
GLYCERIDES—GENERAL

Structure

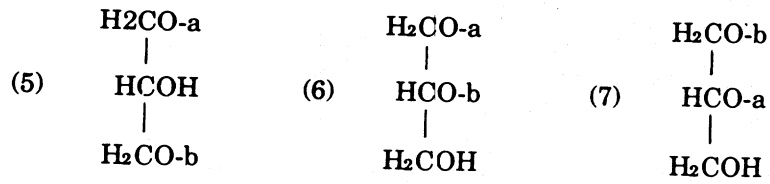
The glycerides dealt with in this chapter are compounds in which 1, 2, or 3 hydroxyl groups of glycerol have reacted to form esters or ethers. Considering the substituents in a general sense as a, b, and c, the various possible isomers are shown below.



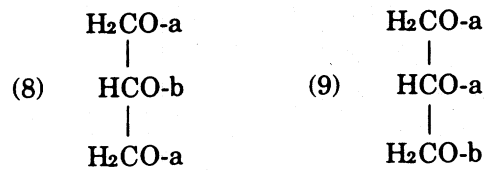
monoglycerides



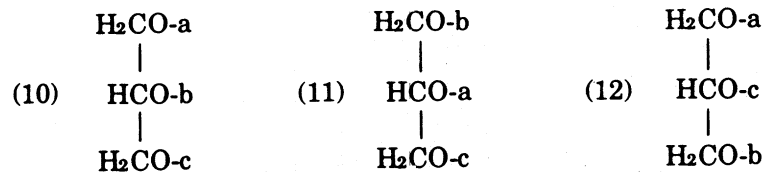
diglycerides with 2 like substituents



diglycerides with 2 unlike substituents



triglycerides with 2 like and 1 unlike substituents



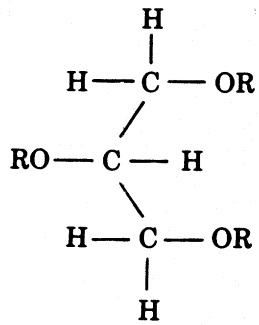
triglycerides with 3 unlike substituents

The positions of attachment of the substituents, from top to bottom, have been variously known as external, internal, external; α, ß, α'; α, ß, α; α, ß, γ; or 1, 2, 3. It is apparent from these examples that the following number of positional isomers are possible: monoglycerides, 2; diglycerides with 2 like substituents, 2; diglycerides with 2 unlike substituents, 3; triglycerides with 3 like substituents (not shown), 1; triglycerides with 2 like and 1 unlike substituents, 2; triglycerides with 3 unlike substituents, 3. Such positional isomers may show differences in physical properties, e.g., melting point, and in chemical properties, e.g., rate of hydrolysis.

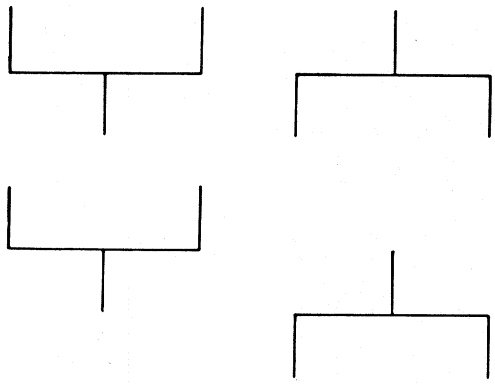
Certain of the above forms are capable of an additional type of isomerism, namely, optical isomerism. Optical isomers are identical in most chemical and physical properties. An exception is their ability to rotate the plane of polarized light; enantiomorphic forms rotate the plane of polarized light an equal amount but in opposite directions. Such isomers also frequently show differences in chemical reactions with other optically active compounds and in those physical, chemical, and biological reactions in which adsorption on an optically active surface is an essential feature. The structural requirement for optical isomerism is an asymmetric molecule. Of the structures given above, 1, 4, 5, 6, 7, 9, 10, 11, and 12 satisfy this requirement, so each of these should be capable of existing in enantiomorphic forms. Optically active mono- and diglycerides were synthesized in early work. However, except for those containing an optically active acid, fatty acid triglycerides with demonstrable optical activity could neither be isolated

from natural sources nor synthesized.[80] Proceeding on the hypothesis that this resulted from a combination of high molecular weight and insufficient disparity between the terminal acyl groups, Schlenk[285] was able to synthesize a number of triglycerides with easily demonstrable optical activity.

The triglycerides are represented above as having all the substituents lying on the same side of the glyceryl group. Actually, in the liquid state there is considerable freedom for groups to rotate about their bonds. In crystals, however, the orientation would be organized and uniform for a particular crystal form. With reference to fatty acid triglycerides, Clarkson and Malkin[48] have concluded from molecular models that the presence of all three fatty acid radicals on the same side of the glyceryl group would introduce considerable strain through steric interference. They proposed instead a "tuning fork" arrangement of the fatty acid groups:

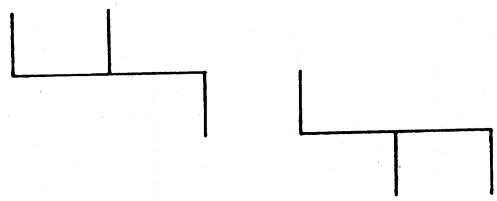


This arrangement is also consistent with the x-ray data showing the length of the triglyceride unit cell to correspond to a double-[48] and sometimes to a triple-chain[46,79,209] structure:



and

Lutton[209] believed that, in certain instances, the triple-chain glyceride was probably represented by a "chair" structure,



rather than by the "tuning fork" structure.

He found both double- and triple-chain structures for the glyceride, C_{18}-C_{12}-C_{18}. Normally a glyceride would appear in only one of these structures; the particular one it appears in would depend upon its fatty acid components. The individual triglycerides can show other differences of molecular packing which produce different crystalline forms. This phenomenon is known as polymorphism. As a result, the unit cells of different polymorphic forms may have different cell dimensions and facial angles and the crystals different physical properties, such as density and melting point. Bailey[21] has stated that at least 2 polymorphic forms have been observed for all fatty acids and glycerides and, for some, as many as 3 or 4. This phenomenon is of interest insofar as it affects the behavior of a fat during melting or solidification. It must also be taken into account in determining the melting point of a pure compound.

The crystalline form obtained from a melt depends upon the temperature at which crystallization takes place. On the basis of existing experimental data, largely on simple saturated triglycerides, it appears that the melt can always be supercooled by rapid cooling to a temperature slightly below the melting point of the least stable form. From these crystals those of intermediate forms, if any, can be obtained by rapidly heating to a temperature only slightly above the melting point of the next higher-melting form, and then cooling. Crystallization from solvents usually results in formation of the most stable form. Transformation of an unstable to a stable form on heating usually occurs at a measurable rate at a temperature appreciably below the melting point of the unstable form. Whether or not this form will be detected during the melting of the material depends upon the rate of transformation and upon the rate of heating. The transformation is slow enough in simple saturated triglycerides to permit easy observation of double melting. In contrast, while two melting points have been obtained for oleic acid, the transformation of saturated fatty acids is so rapid that this phenomenon has never been observed.[21]

Chemical Properties.—The majority of the many chemical reactions which glycerides are capable of undergoing are inappropriate for discussion in this book. Those involved in oxidative deterioration and in the development of lipolytic rancidity are treated in another chapter. Other reactions will be considered together with the lipid classes to which they pertain.

With regard to the various glyceride structures listed above, it was pointed out that some are positional isomers which differ in chemical properties. Although the differences may be small, positional isomers exhibit different reaction rates with either symmetric or asymmetric reagents. A second class of structures is made up of the optically active glycerides which differ from their optical isomers, but only in reactions involving asymmetric reagents. An example of compounds exhibiting this type of chemical differentiation is the monoglyceride, structure 1 above, and its optical isomer in which the substituent "a" is transposed to the other primary hydroxyl group.

There is still a third class of glycerides, namely, compounds which exhibit an internal specificity between like groups in reactions with asymmetric reagents. This class consists of glycerol itself, any triglyceride having 3 identical substituents, and the following glycerides illustrated above: the monoglyceride, structure 2; the diglyceride, structure 3; and the triglyceride, structure 8. All these are examples of the structural type C_{aabc}, which represents a molecule in which the central carbon atom is substituted with 4 symmetrical groups, of which two (a) are identical, but different from the two dissimilar groups (b and c). Such a molecule has a plane of symmetry and therefore is optically inactive. The two halves of the molecule are not identical, however, but have a left hand-right hand relationship. This can be seen in Figure 4.1. Because of the similarity with other compounds, e.g., *meso*-tartaric acid, in which the optical inactivity results from the left hand-right hand relationship of its two halves, the central carbon atom of glycerol and other molecules of the C_{aabc} type has been referred to as a *meso*-carbon atom.[295] In this type of molecule, asymmetric reagents or reactions, such as enzymatically catalyzed reactions which involve intermediate complexes with asymmetric molecules, discriminate between the identical groups.

The possibility of such discrimination was foreseen by Ogston[248] who showed that it could be effected if the reaction involved a 3-point attachment of the substrate to an enzyme. This possibility was later confirmed by Schambye *et al.*[283] and by Swick and Nakao,[296] who demonstrated discrimination between the two primary hydroxyl groups of glycerol in biological systems. Schwartz and Carter[295] showed that this type of discrimination does not require orientation of the substrate

on an enzyme. They reacted 1-α-phenethylamine with ß-phenylglutaric anhydride and found a preferential reaction with one of its two like groups.

The fundamental reason for discrimination between the like groups in C_{aabc}-type molecules by asymmetric reagents might be stated in the generalization that unlike molecules, except optical isomers, differ in energy content and in probability of formation. Reaction of a symmetric reagent with one "a" group (other than a reagent which would alter an "a" group to a "b" or "c" group) forms a mixture of two optically active antipodes. Since the energy content of optical isomers is equal, the mixture contains equal quantities of each antipode. Reaction with an asymmetric reagent, however, produces an unequal mixture of diastereoisomers, since diastereoisomers differ in energy content.

The extent of discrimination in these reactions differs widely. In the Schwartz and Carter reactions with simple compounds, the ratio of diastereoisomers varied from an undetectable difference to 3 to 2 (in the 1-α-phenethylamine-ß-phenylglutaric anhydride reaction). Van Deenen and de Haas[58] hydrolyzed synthetic (α,γ-disteroyl)-ß-lecithin with phospholipase A. In this reaction discrimination appeared to be complete, as one equivalent only of fatty acid was removed to form an optically active lyso lecithin, and no further hydrolysis could be detected after prolonged incubation. In contrast, no discrimination could be detected in the hydrolysis of symmetric triglycerides with pancreatic lipase.[161,338]

To distinguish between its two primary carbinol groups, several systems for numbering the carbon atoms of glycerol have been proposed.

Figure 4.1 illustrates the system in most general current use.[143] Glycerol is oriented with the secondary carbinol group to the observer's

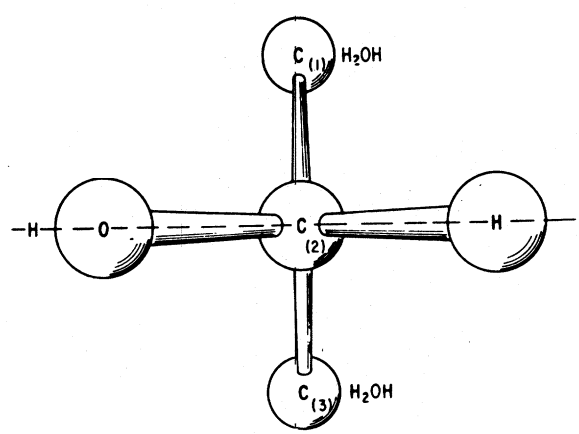


FIG. 4.1. sn-GLYCEROL

left in a Fischer projection in which the substituents above and below the central carbon atom are farther from the observer than those shown to the right and left.[133] The carbon atoms are then numbered as shown in the figure. To distinguish the numbering in this system from that in other systems, the prefix *sn*- designating "stereospecific numbering," is used. This term is printed in the lower case italics and, in names containing glycerol or its equivalent, immediately precedes the term signifying glycerol and is separated from it by a hyphen. For further details the reader is referred to the published recommendations on lipid nomenclature.[143]

Biosynthesis

Before considering the composition of milkfat and its variations with species, diet, and other conditions, it seems appropriate to review information regarding the bio-origins of the substances that are incorporated into the glycerides.

Rumen Activity.—Fatty acids may originate in the lipids of the animal's food or may be formed, in ruminants, by microbial action in the rumen on the nonlipid portion of the diet. To a lesser degree microbial action can alter dietary materials in nonruminants. It is at a minimum in carnivorous species, such as the cat and the dog. The horse and the rabbit represent species with enlarged intestines in which an intermediate degree of activity takes place.

Fatty acids from grass lipids are typically long-chain and contain a high proportion of polyunsaturated fatty acids.[311] Before leaving the rumen the dietary glycerides are largely hydrolyzed to free acids.[92] Microbial activity causes extensive hydrogenation of the unsaturated acids.[93,118,271,311,312] Both saturated and partially saturated acids are formed. The hydrogenation process produces considerable rearrangement of double bonds[4,93,118,307,310-312] both geometrically and positionally, so that one can expect the remaining unsaturated acids to contain an abundance of both geometrical and positional isomers.

Microbial fermentation of dietary carbohydrates in the rumen produces large proportions of acetic acid with smaller proportions of other volatile acids.[8,74] Annison[8] found the following percentage composition of volatile fatty acids in the rumen of sheep which had been fed hay: acetic, 81; propionic, 13; butyric, 4.3; valeric, 0.5; isobutyric, 0.6; isovaleric, 0.3; and 2-methylbutyric, 0.3. The branched-chain acids are derived from dietary protein.[8,10,75]

A number of strains of rumen bacteria require branched-chain volatile fatty acids for growth. Allison *et al.*[5] found that these acids are utilized in forming both branched-chain fatty acids and branched-chain

aldehydes. Isovaleric acid was incorporated into 15-carbon acid, 17-carbon acid, and 15-carbon aldehyde. Isobutyric acid was incorporated into lipids mainly as 14-carbon and 16-carbon fatty acids. Of that incorporated into the lipids, 11% was found in 14- and 16-carbon carbonyl compounds, which presumably were branched-chain aldehydes. The aldehydes were found in the phospholipid fraction and were presumed to be constituents of plasmalogens.

Keeney *et al.*[169] studied the fatty-acid composition of the lipids of the rumen bacteria and protozoa of a cow. The acids were mostly long-chain and included substantial percentages of branched- and odd-carbon acids. The odd- and branched-chain acids were also found in the cow's blood serum lipids, thus showing that they became available to the gland for incorporation into milkfat. The authors concluded, from the nature and percentages of the acids isolated from the microbial lipids, that significant proportions of milkfat acids originate from rumen microbial synthesis of long-chain acids from volatile acids, and that this is the major source of odd-carbon and branched-chain acids.

Circulatory Transport.—Fatty acids that have entered the general circulation, regardless of their origin, are subject to the metabolic requirement of the animal. Utilization in milkfat is but one of these requirements. For example, long-chain acids may be oxidized in the liver to β-hydroxybutyric acid, or may be stored in the animal's depot fat and subsequently be released into the circulatory system over a long period of time. In this connection, conjugated acids have been found in milkfat at elevated levels for a prolonged period after discontinuing the addition of linseed oil to the animal's diet.[134] Likewise, radioactivity from tritium-labeled stearic acid was detected in milkfat for more than a month after feeding.[99]

Volatile fatty acids of the rumen pass directly into the blood and are transported as free acids to the mammary gland by way of the liver. Large quantities of acetic acid reach the gland and are removed by it from the blood. Only a small portion of the propionic and butyric acids, as such, reach the mammary gland. Butyric acid is partly oxidized to β-hydroxy butyric acid by the ruminal epithelia[9,262,340] and both butyric and propionic acids undergo extensive metabolic changes in the liver.[221] Esterified fatty acids, as components of triglycerides, phospholipids, and cholesteryl esters, are carried by the blood to the mammary gland in the form of lipoprotein complexes. Nonesterified medium- and long-chain fatty acids are transported in combination with albumin.

Mammary Gland Activity.—As raw materials for milkfat synthesis, the mammary gland removes both lipids and nonlipids from the blood.

Utilization of Blood Lipids.—It is known that triglycerides are removed from the blood in large quantities and are believed to be a principal source of long-chain acids in milkfat. Albumin-bound nonesterified fatty acids can also be utilized by the gland;[200] it has been postulated that this source of milkfat might become important during fasting. The utilization of fatty acids from other lipid classes is more controversial. This subject is treated in reviews by, for example, Luick[206] and McCarthy.[221] It appears likely that, prior to incorporation into milkfat, the fatty acids of blood glycerides are either hydrolyzed or rearranged.[222,256,259]

The fact that dietary lipids can contribute fatty acids to milkfat has been established by adding unusual fatty acids to the diet and noting their subsequent appearance in the milkfat. Examples are erucic acid from the addition of rapeseed oil to the diet,[131] iodinated fatty acids,[17] tritium-labeled stearic acid,[99] and synthetic odd-carbon acids.[12] Coconut oil cake added to the diet produced elevated levels of myristic and lauric acids in the milkfat.[130] Measurable radioactivity which appeared in the milkfat 4 hr. after feeding tritium-labeled stearic acid[99] could be detected in the fat for 37 days.

The average milk cow secretes about twice as much fat in her milk as she takes in from her diet.[206] This sets an absolute maximum of about 50% for that part of milkfat originating in dietary lipids. Glascock *et al.*,[99] from studies based on the incorporation of radioactive stearic acid, concluded that the actual contribution of dietary fat to milkfat is in the range of 18 to 25%.

Fat Synthesis.—It has been proposed[123] that milkfat is derived entirely from blood fat, the short-chain fatty acids being formed by an *omega* oxidation of fatty acids in the intact triglyceride. It is now known that this view is incorrect. Synthesis of fat in the mammary gland was first recognized from respiration studies and later confirmed with isotopically labeled metabolites. Graham *et al.*[104] found that the respiratory quotient of the lactating goat udder *in vivo* was greater than unity. This indicated utilization of an oxygen-rich substrate for the synthesis of fat. Later, in a series of publications,[85] Folley and French reported their studies of the respiration of tissue slices, taken from the lactating mammary glands of various species, in the presence of glucose or of acetate. The animals studied were mouse, rat, guinea pig, rabbit, sheep, goat, and cow. The ruminant tissue utilized acetate but not glucose, with a respiratory quotient above unity. This situation was reversed with nonruminant tissue. It was concluded that when the respiratory quotient was high, net fatty acid synthesis from an oxygen-rich substrate (acetate for ruminant tissue and glucose for nonruminant tissue) was occurring.

Synthesis of milkfat by the mammary gland has been confirmed

and elucidated by experiments utilizing C^{14}-labeled metabolites. Reviews are available covering much of this research.[82,83,84,261,262] It has been found that acetic acid makes the most important contribution to the synthesis of fatty acids in the mammary gland[275] and is utilized by various species.[53,86,89,146,180,265,266,267] Acetic and butyric acids act as precursors of both fatty acids and glycerol.[275] Although propionic acid is utilized for the synthesis of minor amounts of longer-chain fatty acids with an odd number of carbon atoms,[146] it is relatively unimportant as a precursor of fatty acids.[275] It is, however, indirectly the main glycerol precursor in the cow by conversion into glucose outside the gland.[207,275] Within the mammary gland, glucose is converted into glycerol, which is incorporated into the triglyceride.[207] It appears that valeric acid is utilized for fatty-acid synthesis after being broken down to acetate and propionate.[94] Butyric acid is absorbed into the mammary gland chiefly as β-hydroxybutyrate, which is either reduced directly to butyric acid or first broken down into two-carbon units before participating in fatty-acid synthesis.[9,187,261,305]

The synthetic route by which fatty acids are formed from acetate in the mammary gland has been established by Popják and his associates.[264,265,266] A lactating goat was injected intravenously with $CH_3\ C^{14}OONa$ and the goat milked at frequent intervals up to 48 hr after the injection.[265] It was found that, at the time of their maximum isotope content, the milk fatty acids contained several hundred times as high a concentration of C^{14} as did the plasma fatty acids at any time during the experiment. This indicated that the milk fatty acids must have been synthesized to a large extent within the udder from small molecules and not transferred as such from the blood. The particularly high C^{14} content of the steam-volatile acids indicated that they were synthesized independently rather than being derived from long-chain fatty acids of mammary gland fat. It was calculated that, of the total acetate present in the body of the goat at any time, about 80% would be oxidized within 6 hr, and about 50% of the remainder would participate in the fat metabolism of the udder.

Popják *et al.*[266] resolved the fatty acids obtained from the above-described experiment into two groups: one group contained the acids from all fat samples taken during a period 0 to 12 hr after injection of the isotopically labeled acetate; the other contained acids from samples taken during a 12- to 48-hr period. In the first group the radioactivity per unit of carbon increased to a maximum with decanoic acid, fell off to myristic and palmitic acids and then dropped precipitously to oleic and stearic acids. In the second group the activity increased uniformly to myristic and palmitic acids and then again dropped precipitously to oleic and stearic acids.

These results were interpreted as indicating that in the goat's udder

the intracellular pools of the short-chain acids up to and including decanoic acid are very small, but that they are increasingly larger from lauric acid upward. The very low concentration of C^{14} in stearic and oleic acids indicated that their origin is different from that of the other acids in milkfat. They may be absorbed directly from the blood instead of being synthesized from small molecules. An alternative explanation is that the pools of these acids in the mammary gland tissue are so very large that the concentration of any newly synthesized acids would remain small in comparison to that of the pre-existing acids.

Acetic, butyric, caproic,[266] and caprylic[264] acids were degraded by methods which permitted measurement of the radioactivity of each carbon atom of each acid, together with positive identification of the position within the acid from which the sample was derived. The investigation of acetic acid showed that no redistribution of the C^{14} isotope occurred in the body, since only the carboxyl carbon contained this isotope.

In the other acids, each of the odd-numbered carbon atoms (designating the carboxyl carbon as 1) was radioactive and each of the even-numbered carbon atoms was nonradioactive. Carbon atoms 1 and 3 of butyric acid had equal radioactivity. Carbon atoms 3 and 5 of caproic acid had equal radioactivity, and this was the same as that of carbon atoms 1 and 3 of butyric acid. Carbon atom 1 of caproic acid had a radioactivity approximately 2.5 times that of carbon atoms 3 and 5. These results demonstrate conclusively that butyric acid is synthesized by the combination of two acetate units, and that the synthesis of caproic acid involves the addition of an acetate unit to the carboxyl end of butyric acid. It was likewise shown[264] that synthesis of caprylic acid involves the addition of an acetate unit to the carboxyl end of caproic acid.

The lower activity of the carbon atoms derived from butyric acid was interpreted as indicating that the butyric acid synthesized from acetate was diluted 2.5 times with butyric acid or a butyric acid derivative present in the blood.[266] This compound was not identified, but was believed to be β-hydroxybutyric acid. Such a dilution of the acetate-derived butyric acid was not invariably found with other animals. The fact that in the lactating goat the butyric acid was labeled by having a lower C^{14} content than the acetate provided the basis for the unequivocal demonstration that synthesis always involves the addition of an acetate unit to the carboxyl end of the acid that is being lengthened. Without this circumstance the evidence would not have precluded condensation of larger units nor combination of the carboxyl end of the acetate with the hydrocarbon end of the acid undergoing enlargement.

This synthetic pathway, as described, is incomplete in the sense that a chain elongation by one acetate unit actually involves a series of steps. These intermediate reactions and their requirements of enzymes and cofactors are treated in several general reviews.[52,105,210,353] The review of Folley and McNaught[86] deals more specifically with synthesis by the mammary gland.

At least two distinct pathways may be followed in the biosynthesis of saturated fatty acids. Additional pathways, or at least different enzyme systems, are indicated in some instances. The first step always seems to be the activation of the fatty acids[183] (both components involved in the elongation) by conversion to the thiol ester of coenzyme A:

$$RCOOH + CoASH + ATP \rightleftharpoons RCOSCoA + AMP + PP_i^*$$

The energy necessary for this reaction is furnished by the cleavage of adenosine triphosphate to adenosine monophosphate and inorganic pyrophosphate.

One of the pathways is a reversal of that followed in the *beta* oxidation of fatty acids and probably involves one or more enzymes in addition to those of the *beta*-oxidation sequence.[301] Wakil[353] has suggested that this pathway may be followed in the further elongation of such long-chain acids as palmitic. The steps in a reversal of *beta* oxidation are:

(1) $RCOSCoA + CH_3COSCoA \rightleftharpoons CoASH + RCOCH_2COSCoA$
(2) $RCOCH_2COSCoA \rightleftharpoons RCHOHCH_2COSCoA$
(3) $RCHOHCH_2COSCoA \rightleftharpoons RCH{=}CHCOSCoA$
(4) $RCH{=}CHCOSCoA \rightleftharpoons RCH_2CH_2COSCoA$

While these are equilibrium reactions the equilibrium for step 1 is extremely unfavorable to synthesis. However, an enzyme not involved in the *beta*-oxidation sequence, which can irreversibly reduce the unsaturated ester in step 4, has been discovered.[198,301] This reduction leads, through each of the four steps, to a continuous removal of the product in step 1, making the overall reaction possible.

Another pathway utilizes malonyl coenzyme A which is formed in the first step[352] of the synthesis:

$$CH_3COSCoA + CO_2 + ATP \xrightarrow{Mn^{++}} HOOCCH_2COSCoA + ADP + P_i$$

This reaction is catalyzed by an enzyme containing tightly bound biotin. The major products of the overall synthesis using the enzyme system

*Abbreviations used: CoASH or CoA, coenzyme A; TPNH and TPN+, reduced and oxidized forms of triphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; P_i, inorganic phosphate; PP_i, pyrophosphate.

isolated from pigeon liver can be represented as being formed in accordance with the following equation:

$$RCOSCoA + 7HOOCCH_2COSCoA + 14\ TPNH + 14\ H^+ \rightarrow$$

$$R(CH_2)_{14}COOH + 14TPN^+ + 7CO_2 + 8CoASH + 6H_2O$$

When malonyl CoA was reacted with acetyl CoA, palmitic acid formed 80% of the synthesized acids. When propionyl CoA was substituted for acetyl CoA, the synthesis was much slower and heptadecanoic acid formed 70% of the synthesized acids. When butyryl CoA was used, the reaction rate was even slower; stearic acid formed 85% and palmitic acid 10% of the synthesized acids.

The exact mechanism of this chain elongation and the nature of the intermediate reductive steps have not yet been established. On the basis of available evidence the following scheme has been proposed[35] for the synthesis of long-chain fatty acids by the soluble pigeon liver system:

(1) Both malonyl CoA and acetyl CoA (when the synthesis is started with acetic acid) become bound to the enzymes of fatty acid synthesis by an exchange reaction in which coenzyme A is freed and thiol esters are formed with enzyme sulfhydryl groups.

(2) The enzyme-bound acyl groups undergo a decarboxylation-condensation reaction, which may be followed either by reduction, dehydration, and reduction again to form an enzyme-bound saturated C_4 compound, or by repeated condensations of multiple malonyl groups on the acetyl moiety to form an enzyme-bound keto compound. The carbonyl groups of the ketoacyl compound are then reduced by TPNH, dehydrated, and reduced again to the saturated acyl derivative, which is deacylated to the free fatty acid. In the absence of TPNH, the ketoacyl compound may be deacylated and set free without reduction.

While the mammary gland enzymes are incompletely characterized, there is some evidence that the malonyl-coenzyme A pathway is an important route for the synthesis of fatty acids in the gland. Ganguly[91a] found, using a soluble enzyme system isolated from cow mammary gland, that the rate of synthesis from malonyl CoA is much faster than from acetyl CoA. The C_4, C_6, C_8, C_{10}, C_{12} acids, and a group of longer-chain acids which Ganguly did not resolve, were synthesized by this enzyme system. Dils and Popják[62] studied the synthesis of fatty acids by a soluble enzyme system prepared with rat mammary gland. They concluded that the major route for the synthesis of fatty acids is by way of malonyl coenzyme A. The main products of synthesis with this preparation are the even-numbered fatty acids from C_8 to

C_{18}. These results, in contrast with those from nonmammary tissue enzymes, showed the synthesis of significant amounts of shorter-chain fatty acids.

Avena and Kumar[15] studied the formation of fatty acids by mammary gland slices from the rabbit and the goat. The C^{14}-labeled metabolites studied were acetate, butyrate, and β-hydroxybutyrate. The synthesized acids were isolated as their coenzyme A derivatives and separated into two groups: C_8 and over, and the shorter-chain acids. With both species acetate was incorporated to a greater extent in the longer-chain acids than in the shorter-chain acids; no butyrate was formed from acetate by rabbit slices and none initially by goat slices. About 90% of the β-hydroxybutyric acid incorporated into the fatty acids appeared in the short-chain group. Butyric acid, likewise, but to a smaller extent, was incorporated preferentially in the short-chain acids. These studies showed that in the ruminants, butyric acid and longer-chain acids may be synthesized by two independent processes. Acetate is used to a great extent for chain elongation, while β-hydroxybutyrate is converted predominantly to butyric acid.

It is known that triglycerides are synthesized in the mammary gland from fatty acids and glycerol.[207] The following pathway has been established for this synthesis in nonmammary gland tissues:

(1) $2RCOSCoA + CH_2OHCHOHCH_2OPO_3^{=} \rightarrow$
$CH_2O(OCR)CHO(OCR)CH_2OPO_3^{=}$

(2) $CH_2O(OCR)CHO(OCR)CH_2OPO_3^{=} \rightarrow$
$CH_2O(OCR)CHO(OCR)CH_2OH$

(3) $CH_2O(OCR)CHO(OCR)CH_2OH + RCOSCoA \rightarrow$
$CH_2O(OCR)CHO(OCR)CH_2O(OCR)$

In the first step two molecules of fatty acids, in the active form of coenzyme A esters, react with L-α-glycerophosphate to form a phosphatidic acid.[184] In the second step this is converted to an α,β-diglyceride by the action of a specific phosphatase.[320] The diglyceride is then esterified by a third fatty acyl CoA to form the triglyceride.[356]

The synthesis of triglycerides by mammary gland enzymes has been studied by Kumar and Pynadath,[188] using a mitochondrial-microsomal particulate fraction from lactating goat mammary glands. The glyceride synthetic activity resides in this fraction. C^{14}-labeled palmitate and palmityl CoA are incorporated into glycerides in proportions comparable to those found in intestinal mucosa, adipose tissue, and liver. Besides palmityl CoA, octanyl CoA and hexanoyl CoA are incorporated in relatively large percentages. Butyryl CoA is incorporated to a much lesser extent than in goat milkfat. The authors were unable

to explain this. The fatty acid acceptors are α-glycerophosphate and 1,2-diglycerides. The results are in agreement with the scheme proposed by Weiss *et al.*[356]

Unsaturated Acids.—Most of the experimental material cited in this section does not establish the body sites where unsaturated acids are formed. Even though synthesized outside the mammary gland, they may be transported in the general circulation to the gland and thus become available for incorporation into milkfat.

There is no proof that unsaturated acids can be formed in animal tissues by biohydrogenation of more highly unsaturated acids. C^{14}-labeled palmitoleic acid fed to rats was converted in small amounts to radioactive palmitic acid.[227] The extent of this biohydrogenation was of relatively minor importance; the possibility that even this occurred in the intestines from microbial action could not be excluded. In contrast, it has been demonstrated that, by dehydrogenation, double bonds can be introduced into saturated acids and into certain unsaturated acids.

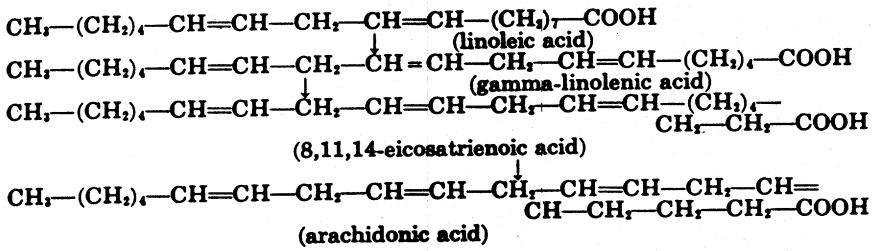
Schoenheimer and Rittenberg fed mice the methyl esters of linseed oil which had been reduced catalytically with deuterium.[287] The unsaturated acids from the body carcass fat contained more deuterium than the body water. Stetten and Schoenheimer,[329] by isotopic exchange, prepared a palmitic acid containing 22 atom per cent deuterium. This was fed to rats as the methyl ester. The unsaturated acids, after isolation from the acids of the carcass fat, were hydrogenated. Palmitic acid containing deuterium was found. This demonstrated a desaturation of palmitic acid. In a similar experiment[7], in which C^{14}-labeled myristic acid was included in the diet of rats, desaturation of myristic acid was demonstrated.

These experiments are sometimes cited as demonstrating the formation of oleic, palmitoleic, and myristoleic acids from their saturated counterparts. They did not, in fact, show either the number or the position of double bonds introduced into the saturated acids. Glascock and Reinius[100] provided more definite evidence on these points. Tritium-labeled stearic acid was fed to a goat and oleic acid was isolated from the milkfat after conversion by oxidation to a characteristic derivative, dihydroxystearic acid. The activity of this derivative was such as to indicate that it was derived from an oleic acid which had been formed by desaturation of the labeled stearic acid.

Lauryssens *et al.*[200] have subsequently shown that desaturation can take place in the mammary gland. More than one-half of the activity incorporated into udder tissue glycerides from 1-C^{14}-stearate in a perfusion experiment was found in the oleic acid fraction of the chromatographically separated fatty acids.

Enzyme systems and pathways in the formation of monounsaturated acids in yeasts[26] and in an anaerobic bacterium[284] have been discussed by Bloch and his associates.

Mead, in a review article[225], has made a remarkably clear presentation of the principles underlying the formation of polyunsaturated fatty acids in animals. The steps in the conversion of linoleic to arachidonic acid are shown below:



This pathway was elucidated by a series of experiments[140,226,228,327] involving the administration to rats of C^{14}-labeled acids, followed by determination of the location of the C^{14} in arachidonic acid. Following the administration of acetate-1-C^{14}, activity appeared only in the carboxyl group of arachidonic acid. Linoleate-1-C^{14} activity appeared in carbon 1, as a result of its breakdown to acetate, and in carbon 3. Gamma-linolenate-1-C^{14} activity appeared almost exclusively in carbon 3, indicating a very rapid conversion to arachidonic acid. The activity from 8,11,14-eicosatrienoic-2,3-C^{14} acid appeared in carbons 2 and 3 of arachidonic acid.

It was likewise shown[181,224,328] that linolenic acid is converted by a series of similar steps to the end products, 5,8,11,14,17-eicosapentaenoic acid, 7,10,13,16,19-docosapentaenoic acid, and 4,7,10,13,16,19-docosahexaenoic acid. Oleic acid was shown[90] to be the precursor of 5,8,11-eicosatrienoic acid in fat-deficient rats. A pathway analogous to those from linoleic and linolenic acids was indicated both by the constitution of the trienoic acid itself, and by the isolation of 8,11-eicosadienoic acid, a postulated intermediate. In this same study another triene, 7,10,13-eicosatrienoic acid, was isolated. This acid presumably would be formed in a similar manner from palmitoleic acid.

These results can be generalized[225] as follows: Starting from the existing double bonds of the precursor acid, additional double bonds are introduced in a 1,4-relationship toward the carboxyl group. This dehydrogenation is limited by the restriction that a double bond cannot be introduced closer to the carboxyl than the 4-position. Chain lengthening by the addition of acetate units may provide one or more intermediate precursors which can be dehydrogenated further.

It is notable that each of the initial precursor acids in these studies has a *cis* double bond in the 9-position. Intermediates, with *cis* double bonds nearer the carboxyl, but with definite relationships to the 9-position as shown above, also served as precursors of more highly unsaturated acids. Holman[136] has shown that *trans* isomers can be dehydrogenated. If the positional isomer of oleic acid, *cis*-12-octadecenoic acid, could serve as a precursor, it would be converted initially to linoleic acid; but it has been reported to be ineffective as an essential fatty acid.[344]

Mead[225] has suggested that the formation of polyunsaturated acids requires three enzyme systems: a polydehydrogenase which dehydrogenates mono- or polyenoic acids in a 1,4-relationship from the existing double bonds toward the carboxyl group; an acyl transferase, which adds two carbons when chain-lengthening is necessary; and a system which disposes of unsaturations in the 2- or 3-positions.

Mead has also discussed the special physiological functions of the polyunsaturated acids in relation to their shapes and polar centers. Kishimoto and Radin[178] determined the structure of a number of monoenoic and several dienoic acids isolated from brain sphingolipids. The chain lengths and positions of unsaturations of some of these acids indicated the likelihood that they were formed, via an α-oxidation system, by the removal of one carbon atom from the next higher homolog.

NEUTRAL GLYCEROLIPIDS

Fatty Acid Triglycerides

Chemical Properties.—Pertinent reactions of the ester linkage can be generalized as follows:

$$G{-}O{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}R + R'\,OH \rightleftharpoons R{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}O{-}R' + G{-}OH$$

When R′ is hydrogen, the hydrolysis forms fatty acids and, successively, diglycerides, monoglycerides, and glycerol. The reaction with water alone takes place slowly at room temperature and, in the presence of both reaction products, is incomplete. It can be speeded at elevated temperatures and in the presence of a catalyst. The reaction velocity is also affected by certain substituents in the acyl moiety of the glyceride. It can be driven to completion by removing either of the reaction products as formed.

Fatty acids with an odd number of carbon atoms were reported to

be liberated from stored butter more rapidly than acids with an even number of carbon atoms.[166] The increase in free fatty acids in sterile concentrated milk during storage appeared to result from random hydrolysis of the milk lipids.[203]

When R′ is an alkyl group, the acyl groups of the glyceride equilibrate between the hydroxyls of the glycerol and those of the added alcohol. If a suitable catalyst is used, e.g., an alkali-metal alcoholate with a sufficient excess of anhydrous alcohol, the reaction is complete enough to serve as a practical means of obtaining esters without the more laborious intermediate step of isolating the free acids. If the glyceride is heated and another ester and a catalyst, such as sodium ethylate or sodium-potassium alloy, or with the catalyst alone, the resultant reaction is known as interesterification; it consists of random rearrangement of the acyl groups among all the hydroxyl groups.

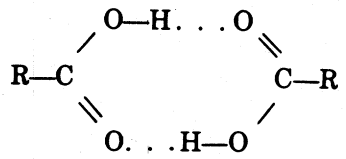
General Properties of Fatty Acid Components.—About 85% of the total weight of the milk lipids occurs in the fatty acid moiety of the triglycerides. The properties of milkfat, accordingly, are influenced by those of their component acids and by the relative proportions and the inter- and intramolecular distribution of these acids.

The lower molecular weight saturated acids and the 9-*cis* unsaturated acids up to the C_{20} acid, which melts at 25°C, are liquids at normal room temperatures. Factors affecting the melting point include the molecular weight, the number and position of alkyl substituents, and the number, position, and geometrical configuration of unsaturations.

The series of fatty acids with an even number of carbon atoms and that with an odd number both show a nearly linear increase of melting point with increased molecular weight, but the curve of the latter is displaced downward on the melting-point scale. The melting point of an acid is also a function of its degree of symmetry. For a given molecular weight the melting point is highest for the straight-chain isomer. The melting point is progressively lowered with increased branching, and the effect is the greatest for branching located near the center of the molecule.

The boiling points of fatty acids also increase with increased molecular weight, but without distinction as to whether the acid has an odd or an even number of carbon atoms. The boiling point of an acid decreases with increased symmetry and with increased unsaturation, but the effect is much smaller than in the case of the melting point. Treatises are available[60,216] covering these and other physical properties of the fatty acids.

The fatty acids are among those compounds which can associate by means of hydrogen bonding. In this instance a dimer is formed:



X-ray analysis showing crystals having layers two molecules thick has substantiated this structure. This double-molecule structure has the effect of raising the melting point of fatty acids considerably above values found for nonassociated compounds of equal molecular weight. The ester derivatives of fatty acids are, for the most part, unassociated, and consequently have considerably lower melting points than acids of the same molecular weight. For example, the melting point of stearic acid has been reported as 69.6°C, of ethyl stearate as 34.0°C, and of ethyl palmitate (same molecular weight as stearic acid) as 25.0°C. The methyl esters are apparently partially associated. This would account for the fact that, in general, they have somewhat higher melting points than the corresponding ethyl esters. The effect of polymorphism on the melting point of fatty acids has been mentioned in the section on glycerides.

Hydrogen bonds are weakened with an increase in temperature. At the boiling points of formic and acetic acids they are still strong enough to maintain the integrity of the dimeric form. This phenomenon causes the fatty acids to boil at higher temperatures than unassociated molecules of the same molecular weight. It is, therefore, common practice to use the methyl or ethyl esters in effecting separations of the fatty acids by distillation. It is also common practice to use ester derivatives in effecting separations of the fatty acids by gas-liquid chromatography. Separations of the free acids are best accomplished with a polar stationary phase. Under these conditions association of the acids, with its resultant complications, is minimized.

Contrary to a widespread belief, the fatty acids do not ionize appreciably less as their molecular weight increases above that of butyric acid.[172] This is of interest in connection with the titration of acids. If one considers the following equilibrium,

$$RC(=O)OH + OH^- \rightleftharpoons RC(=O)O^- + H_2O,$$

he will find that, regardless of molecular weight, the fatty acids can be determined quantitatively by titrating to a suitable end point if the reactants are maintained in solution. It is well known that the salt of a higher fatty acid hydrolyzes to a considerable extent in water to give an alkaline solution, e.g.,

$$C_{17}H_{35}\overset{\overset{\displaystyle O}{\|}}{C}{-}O^- + H_2O \rightleftharpoons C_{17}H_{35}\overset{\overset{\displaystyle O}{\|}}{C}{-}OH + OH^-.$$

This reaction is not primarily due to a low ionization constant of stearic acid, however, but rather to its insolubility in water. The alcoholic alkali solution generally used for determining fatty acids provides sufficient solvent action to prevent excessive hydrolysis.

Determination of Fatty-acid Composition.—Hilditch[124] has described the methods by which he and his associates, as well as others, accumulated much of the earlier data on the fatty-acid composition of triglycerides. The methods employed were aimed at separating the complex mixture of fatty acids into groups containing no more than two acids each. Steam distillation of the acids, fractional vacuum distillation of the esters, and differential solubilities of the salts, chiefly the lead salts, were utilized toward this end. Determinations of the saponification and iodine values, and the weights of material in each fraction, gave the information needed for a quantitative estimation of the acid composition of the fat. If the preliminary separations failed to limit a fraction to two acids, as often occurred in the group of C_{18} acids, additional separations or analyses were necessary.

Chromatographic methods have proved to have outstanding utility in recent investigations of fatty-acid composition. Adsorption chromatography has been applied successfully[190,274] to the difficulty separable group of C_{18} acids. This form of chromatography has been largely superseded, however, by partition chromatography, both liquid-liquid and gas-liquid. Other useful techniques are low-temperature (down to −65 to −70°C) crystallization from solvents and countercurrent distribution. Of these various techniques low-temperature crystallization is probably the least effective in separating individual fatty acids. It has the advantage of being applicable, without damage to the fatty acids, to large quantities of material. These characteristics have made it useful, as a preliminary step, in obtaining concentrated samples of a particular type of acid, e.g., the polyethenoic acids. Fractional vacuum distillation is also applicable to large samples of fatty acids (usually as the methyl esters). It is effective in separating acids on the basis of chain length—much less so on the basis of unsaturation. It has the disadvantages of being relatively slow and, as a result of prolonged exposure to elevated temperatures, of subjecting the acids to possible thermal damage. Countercurrent distribution is applicable to samples of intermediate size. The apparatus is rather bulky and the procedure is time-consuming. However, the material is not damaged, and it is sometimes possible to obtain effective separations by

proper selection of solvents and application of a sufficiently large number of equilibria.

Chromatographic methods, particularly those employing gas-liquid chromatography, permit the use of columns with very high theoretical plate ratings. An interesting example of the high efficiency of gas-liquid chromatography is the resolution of the four geometrical isomers of methyl linoleate.[202] In general, the amounts of material separable by gas-liquid chromatography are small, although a distinction is made between preparative and nonpreparative columns. By using the former and by combining material collected from identical portions of a series of separations, samples of sufficient size for further investigation can be obtained. The elevated temperatures employed in gas-liquid chromatography may cause structural alternations in certain esters.[233] Conjugated trienoates can be isomerized geometrically, and esters with a hydroxyl or hydroperoxide group *alpha* to a double bond can undergo dehydration. In general, other types of acid are unaffected.[233,332] This technique requires expensive equipment and considerable experience in order to avoid misinterpretations of the data. Columnar liquid-liquid chromatography, in contrast, is an elegantly simple method, in regard to both the required apparatus and its operation, and is suitable for larger samples. Paper and thin-layer chromatography are modifications adapted to the resolution and quantitative estimation of small samples. The required apparatus and its operation are both very simple. These methods are eminently suited to the routine analysis of a large number of samples.

The methods employed in identifying and estimating the acids in the various fractions separated from a fat depend upon the complexity of its fatty acid composition and upon whether or not acids of unknown structure are present. In the simpler situations it may be sufficient to determine the weight, saponification value, and iodine value of a distillation fraction, the position and area under a peak in a gas chromatogram, or the position and titration value of a fraction obtained by columnar partition chromatography. In more complex situations additional tests or procedures are necessary. Reviews are available on the investigation of fatty acid structure by chemical means,[326] by nuclear magnetic resonance,[138] mass spectrometry,[71] infrared spectroscopy,[246] and ultraviolet spectroscopy.[245]

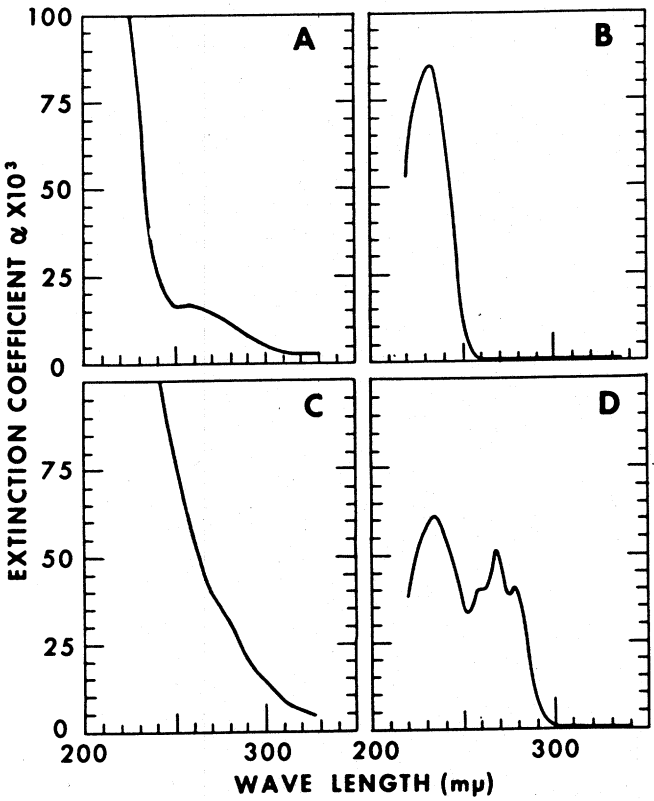
In absorption spectra the following relation exists between the wavelength of absorbed radiation and the energy change involved in its associated transition: $\Delta E = hc\lambda$. In this equation E is the absorbed energy, h is Planck's constant, c is the speed of light and λ is the wavelength of absorbed radiation. It is apparent that the energy change becomes smaller as the wavelength increases.

Ultraviolet absorption spectra arise from transitions of valence electrons between different energy levels. Fatty acids with isolated double bonds show absorption maxima in the region just below 200 mμ. Conjugation introduces transition levels with smaller energy differences, thus shifting the absorption maxima to longer wavelengths. Two conjugated double bonds shift the absorption band to about 234 mμ, 3 conjugated double bonds to about 268 mμ, 4 to 315 mμ, 5 to 346 mμ, 6 to 374 mμ, etc. Compounds with a sufficiently large number of conjugations, e.g., carotene, absorb in the region of longer wavelengths associated with visible light, and are therefore colored. On the basis of data obtained from known compounds, the absorption spectra of solutions can be used to detect the presence of conjugated acids and to estimate their concentrations from, respectively, the positions of absorption maxima and their intensities.

Alkali isomerization rearranges the isolated double bonds into a conjugated system. Under standardized conditions the absorption spectra before and after isomerization afford data suitable for determining the identity of nonconjugated acids (with respect to the number of double bonds present) and their concentrations. Fig. 4.2 shows the ultraviolet absorption spectra of linoleic and linolenic acids both before and after alkali isomerization. It is to be noted that isomerized linolenic acid shows absorption maxima for systems of both 3 conjugated double bonds and of 2 double bonds. The latter, of course, is part of the total system. The determination of unsaturated acids by ultraviolet absorption spectroscopy is described in detail by Mehlenbacher.[229]

Absorption in the infrared region concerns radiation of considerably longer wavelengths than that of the ultraviolet, and accordingly involves much smaller energy changes. These spectra involve vibrations of atoms or groups between different energy levels within a molecule. O'Connor[245] has assembled a collection of infrared absorption bands applicable to fatty acids and other lipids. Absorption at appropriate wavelengths is evidence of the presence of definite groups. Fig. 4.3 illustrates the distinction between absorption due to *cis* double bonds and that characteristic of *trans* double bonds. The latter has been developed into a quantitative determination.[247] Many additional structural features can be recognized from the presence of absorption at specific wavelengths. Comparison of infrared spectra with those of known compounds is an effective means of establishing the identity of unknown compounds or of those whose identity is doubtful. Infrared absorption spectroscopy has become an important tool in lipid research.

Spectroscopic data on lipids are usually reported as the wavelength (λ) in angstroms (A), millimicrons (mμ), or microns (μ), or as wavenumbers, i.e., the number of waves per unit length. For absorption



Adapted from O'Connor[344]

FIG. 4.2. ULTRAVIOLET ABSORPTION SPECTRA OF POLY-UNSATURATED FATTY ACIDS BEFORE AND AFTER ALKALI ISOMERIZATION

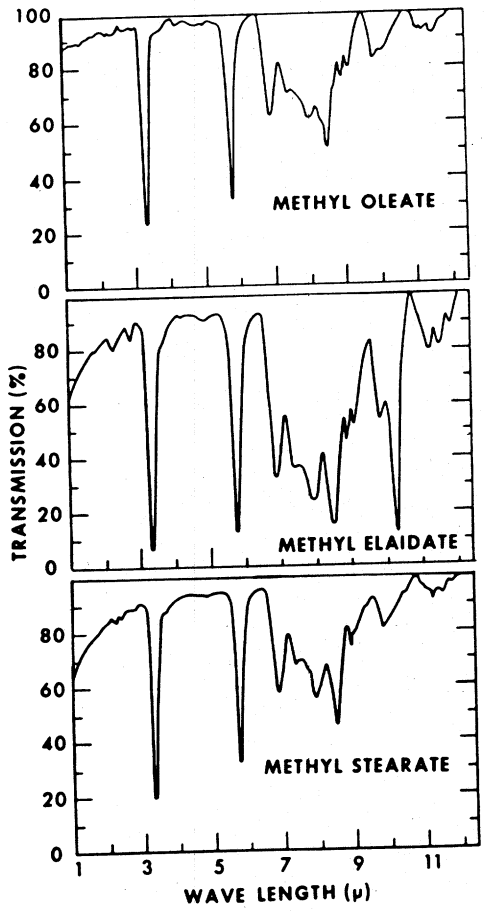
A—Linoleic acid before. B—After. C—Lineolic acid before. D—After.

in the infrared region it is usually expressed in reciprocal centimeters (cm^{-1}). The relationships between these units are given here for the convenience of the reader who may need to interconvert data for the sake of making comparisons in the same units:

1 meter = 10^2 centimeters = 10^6 microns = 10^9 millimicrons = 10^{10} angstroms

10^4/(wavenumber in cm^{-1}) = wavelength in microns.

It was stated above that the methods employed in an analysis depend upon the complexity of the material. It should be emphasized here that they also depend upon the purpose of the investigation, which may, for example, be satisfied with a determination of only the known major component acids. In this instance a simple analytical procedure



Adapted from Feuge, Pepper, O'Connor and Field[78]

FIG. 4.3. COMPARISON OF INFRARED ABSORPTION SPECTRA OF *cis*-UNSATURATED, *trans*-UNSATURATED, AND SATURATED FATTY ACID ESTERS

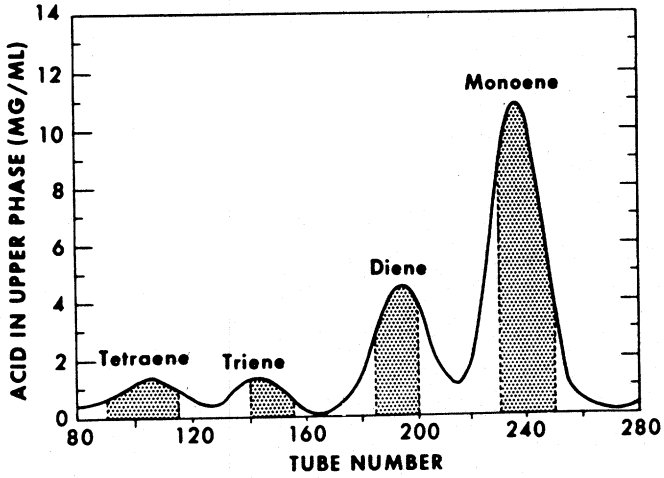
night well be adequate. This section is being concluded, however, with an illustration of the use of various techniques in the investigation of a complex oil in which it was felt necessary to obtain positive identification of the component acids.

In this investigation[331] it was desired to characterize completely and with certainty the unsaturated fatty acids of menhaden body oil. The acids from 500 gm of oil were fractionally crystallized from acetone at temperatures ranging down to −70°C. Those soluble at this temperature (137 gm) were esterified with methanol, and 100 gm of esters was fractionally vacuum-distilled. The fractions rich in C_{16} acids were

combined and redistilled into 9 fractions. Small samples of each fraction were hydrogenated and checked for homogeneity by gas-liquid chromatography. Fractions 4 to 7 were exclusively C_{16}. Their ultraviolet spectra showed no conjugated acids; after isomerization of small samples the presence of dienoic, trienoic, and tetraenoic acids was shown.

Six gm of C_{16} esters, free from other chain lengths, was saponified and the free acids separated by countercurrent distribution into groups of acids differing in number of double bonds. Using a 200-tube apparatus and 375 equilibria, the acids were separated into 4 groups, as shown in Fig. 4.4. Aliquots of each peak were isomerized with alkali. Ultraviolet spectra showed the identity of each peak in terms of the number of double bonds. These spectra also showed that each peak was free from contamination by more highly unsaturated acids. Superimposition of these experimental curves on theoretical curves furnished additional evidence of purity. Ultraviolet analysis of the unisomerized material showed the absence of conjugated acids, and infrared analysis the absence of *trans* isomers.

Each of these peaks was thus homogeneous with respect to chain length, to the number of double bonds, and to geometrical isomers, but might contain positional isomers needing characterization of the double bond patterns. This was accomplished as follows: Oxidative ozonolysis degraded the unsaturated acids by attack at the double



Adapted from Staffel and Ahrens[331]

FIG. 4.4. SEPARATION BY COUNTERCURRENT DISTRIBUTION OF UNSATURATED FATTY ACIDS ON BASIS OF NUMBER OF DOUBLE BONDS

System: heptane/methyl alcohol, formamide, acetic acid.

bonds into mixtures of mono- and dicarboxylic acids. Utilizing columnar, paper, and gas-liquid chromatography, these fragments were identified and quantitated. From these data it was possible to identify the original structures. The presence of terminal unsaturations was verified by infrared spectra showing absorption maxima at 910 and 990 cm^{-1}.

Techniques additional to those mentioned above have been and are being made available for lipid research. One of particular utility is the use of silica gel or silicic acid modified by impregnation with silver nitrate. This adsorbent has been widely used in both columnar and thin-layer partition chromatography, e.g., for separating *cis*- from *trans*-fatty acid isomers,[61] methyl esters of different degrees of unsaturation,[178] and conjugatable from *cis, cis*-nonconjugatable dienoates.[156] Mass spectrometry is now used in many structural investigations. In determining the structure of the hydroxy acid components of milkfat sphingolipids, Morrison and Hay,[236] prior to mass spectrometry, converted the acids to the trimethylsilyl ethers. The resultant spectra, unique for ethers in the 2-position, identified the structure of the hydroxy acids unambiguously.

Component Fatty Acids of Cow's Milkfat.—Prior to 1951, the fatty acids found in natural glycerides had been characterized, almost without exception, by a straight chain and an even number of carbon atoms. The isolation by Hansen and Shorland[110] of 14-methylhexadecanoic acid and 15-methylhexadecanoic acid from milkfat provided acids having neither of these characteristics. These were but the first of what has become a long series of branched-chain and odd number-carbon atom acids isolated from natural fats. The following branched-chain saturated acids have also been reported to be present in milkfat: 10- and 11-methyldodecanoic acids,[309] 12-methyltridecanoic acid,[116] 12- and 13-methyltetradecanoic acids,[115,308] 14-methylpentadecanoic acid,[114] a methylheptadecanoic acid of uncertain structure,[113] and a multibranched C_{20} acid of uncertain structure.[111] Such an acid has recently been identified[23,324] as 3,7,11,15-tetramethylhexadecanoic acid. It is noted that most of these acids are branched on either the penultimate or the antepenultimate carbon atom. Those with branching on the antepenultimate carbon atom have structures meeting the essential requirement for optical activity. In each instance the form isolated has been the dextrorotatory isomer.

Unsaturated Acids.—Oleic, the most abundant of this group, is *cis*-9-octadecenoic acid. C_{10}, C_{12}, C_{14}, and C_{16} acids with a double bond at the 9-position have also been identified.[129,299] The C_{12} and C_{14} acids each had an isomer with a terminal unsaturation; but the positional isomer with internal unsaturation predominated and had both the

cis and *trans* configuration,[211] the *cis* form predominating.[299] The C_{16} and C_{18} acids, likewise, were shown to have both *cis* and *trans* isomers.[299] *Cis*-9-heptadecenoic acid has been isolated from butterfat in a concentration of approximately 0.06%.[117] Vaccenic acid (*trans*-11-octadecenoic acid) was reported by Bertram[24] to be present in milkfat to the extent of 0.01%. More recent investigations have confirmed its presence in substantially higher concentrations.[18,96] Positional isomers (4-octadecenoic acid,[147] 13-octadecenoic acid,[147] *trans*-16-octadecenoic acid,[18] and the series of 7-to 14-octadecenoic acids[164]) have also been reported.

The presence of linoleic acid (*cis, cis*-9-12-octadecadienoic acid) in milkfat has been a subject of controversy. Hilditch and Jasperson[128] failed to obtain tetrabromo- or tetrahydroxystearic acid from the octadecadienoic acid which they identified spectrophotometrically and by other means. From chemical studies they concluded that the acid is *cis*-9, *trans*-12- and/or *trans*-9, *cis*-12-octadecadienoic acid. Shorland,[306] using what he considered a sensitive technique for detecting the tetrabromo-derivative of linoelic acid, also failed to find any evidence of linoleic acid in New Zealand milkfats. These findings differ from those of White and Brown[358] who, after a preliminary concentration of the acid by low-temperature crystallization, obtained the tetrabromo-derivative of linoleic acid in such amounts as would have been obtained if ⅔ to ¾ of the octadecadienoic acid were linoleic acid.

Shorland[306] found that the octadecatrienoic acid content of New Zealand milkfat consists almost entirely of linolenic acid, i.e., *cis, cis, cis*-9,12,15-octadecatrienoic acid. He has suggested that this finding—in contrast with that of Hilditch and co-workers, who could not isolate the hexabromo- derivative of linolenic acid in any appreciable quantity—might be explained on the basis of direct incorporation of linolenic acid present in the diet into the milkfat. Bosworth and Sisson[29] isolated octabromoarachidic acid and concluded that arachidonic acid (*cis, cis, cis, cis*-5,8,11,14-eicosatetraenoic acid) is present in milkfat.

Sambasivarao and Brown[280] have presented impressive evidence, based on the use of a variety of techniques, that both linoleic and linolenic acids are indeed present in milkfat. Fractions containing concentrates of the polyunsaturated C_{18}-acids were analyzed. The linoleic acid content of the dienoic acids averaged about 62%, that of conjugated acids about 10%, and the remainder consisted of *cis, trans* or *trans, cis* isomers of which the greater part consisted of positional isomers with more widely separated double bonds. The linolenic acid content of the C_{18}-trienoic acids averaged 75%.

Since the above material was written for the 1965 edition of this book, the list of known acids has been tremendously expanded. With

only a few exceptions, Tables 4.2 to 4.6 contain all the unsubstituted fatty acids (other than the keto- and hydroxy acids) known by this author to have been reported as constituents of the neutral glycerolipids of cow's milk. The following acids also have been reported: the dibasic acids, glutaric, adipic, pimelic, and azeleic,[38] which were identified on the basis of chromatographic retention data, and 4-octadecenoic acid,[147] which was not found in a recent detailed study of the octadecenoic acids of milkfat.[121]

Identification of the acids involved a number of techniques, among which gas-liquid chromatography was invariably prominent. Because of the complexity of the total fatty-acid mixture, identification always was preceded by treatments designed to simplify the mixtures. These preliminary operations included fractional crystallization, fractional distillation, urea-complex fractionation, columnar chromatography, preparative gas-liquid chromatography, thin-layer chromatography, and argentation chromatography.

In addition to chromatographic retention data, infrared, ultraviolet, and mass spectrometry were used in final identification. Isomeric mixtures of unsaturated acids were separated into *cis* and *trans* groups by means of argentation chromatography, and the double bond positions located by oxidative cleavage followed by chromatographic analysis of the fragments.

Distribution of Fatty Acids in Triglycerides.—The manner in which these acids are associated in the triglycerides has been the subject of a vast amount of research effort. The monoacid or simple triglyceride theory postulated that each triglyceride contains only one kind of acid,

Table 4.2
NORMAL SATURATED FATTY ACIDS OF BOVINE MILKFAT [a]

Number of Carbon Atoms	Wt. %	Number of Carbon Atoms	Wt. %
2	0.09	16	23.8
4	2.79	17	0.70
5	0.01	18	13.2
6	2.34	19	0.27
7	0.02	20	0.28
8	1.06	21	0.04
9	0.03	22	0.11
10	3.04	23	0.03
11	0.03	24	0.07
12	2.87	25	0.01
13	0.06	26	0.07
14	8.94	27	< 0.01
15	0.79	28	< 0.01

[a] Data for C_4 acid from reference 119 and for C_{27} and C_{28} acids from reference 144. All other data from reference 122.

Table 4.3

MONOBRANCHED SATURATED FATTY ACIDS OF BOVINE MILKFAT

Number of Carbon Atoms	Structure	Ref.	Wt. %[a]	Ref.
9	Branched	166	< 0.01	166
11	Branched	166	< 0.01	166
12	9-Methylundecanoic	278	0.1 mole %	278
12	10-Methylundecanoic	144	0.01	144
13	10-Methyldodecanoic	309	0.05 mole %	278
13	11-Methyldodecanoic	309	0.04	144
14	12-Methyltridecanoic	116	0.03	144
15	7-Methyltetradecanoic	278	—	—
15	8-Methyltetradecanoic	278	—	—
15	9-Methyltetradecanoic	278	—	—
15	10-Methyltetradecanoic	278	—	—
15	11-Methyltetradecanoic	278	—	—
15	12-Methyltetradecanoic	115, 308	0.23	94
15	13-Methyltetradecanoic	115, 308	0.14	122
16	14-Methylpentadecanoic	114	0.20	144
17	8-Methylhexadecanoic	278	—	—
17	9-Methylhexadecanoic	278	—	—
17	10-Methylhexadecanoic	278	—	—
17	11-Methylhexadecanoic	278	—	—
17	12-Methylhexadecanoic	278	—	—
17	14-Methylhexadecanoic	110	0.23	144
17	15-Methylhexadecanoic	110	0.36	144
17	11-Cyclohexylundecanoic	288	≥0.01	288
18	16-Methylheptadecanoic	144	0.02	144
19	16-Methyloctadecanoic	278	0.3 mole %	278
20	Branched	122, 144	0.01	144
21	Branched	144	0.01	144
22	Branched	144	0.02	144
23	Branched	144	0.01	144
24	Branched	144	0.02	144
25	Branched	144	0.004	144
26	Branched	144	0.004	144

[a] The seven C_{15} acids comprised 1.20 mole % and the eight C_{17} acids 0.70 mole % of the total acids of the neutral glycerolipids. Data from reference 278.

the number of different triglycerides being equal to the number of acids. Based more on assumption than experiment, this theory is of historical interest only.

Details of earlier studies of glyceride structure have been described by Hilditch.[124] This work, to a large extent, was based on determining the distribution of fatty acids among the main glyceride types based

Table 4.4

MULTIBRANCHED SATURATED FATTY ACIDS OF BOVINE MILKFAT [a]

Number of Carbon Atoms	Structure	Wt. %
16	D,D-4,8,12-Trimethyltridecanoic	0.005
17	3-Side chains	0.01
18	3-Side chains	0.16
19	2,6,10,14-Tetramethylpentadecanoic	0.01
20	3,7,11,15-Tetramethylhexadecanoic	0.12 [b], 0.18 [c]
21	4 Side chains	0.2
22	4 Side chains	0.02
23	4 Side chains	0.01
24	4 Side chains	0.10
25	4 Side chains	0.10
26	3 Side chains	0.01
26	4 Side chains	0.04
27	4 Side chains	0.04
28	3 Side chains	0.02
28	4 Side chains	0.12
28	5 Side chains	0.01

[a] Data for C_{16} acid from reference 108, for C_{19} acid from reference 109, and for C_{20} acid from references 16, 23, and 324. All other data from reference 144.
[b] Spring fat.
[c] Summer fat.

Table 4.5

MONOENOIC FATTY ACIDS OF BOVINE MILKFAT

Acid [a,b]	Structure	Wt. %
10:1	9-Decenoic	0.27
12:1	11-Dodecenoic	0.14
12:1	9-*cis*-Dodecenoic	
12:1	9-*trans*-Dodecenoic	
14:1	5-*cis*-Tetradecenoic	0.0075
14:1	6-*cis*-Tetradecenoic	0.0060
14:1	7-*cis*-Tetradecenoic	0.0068
14:1	8-*cis*-Tetradecenoic	0.0045
14:1	9-*cis*-Tetradecenoic	0.72
14:1	*trans*-Tetradecenoic	0.0076
14:1	13-*trans*-Tetradecenoic	—
15:1	Pentadecenoic	0.07
16:1	5-*cis*-Hexadecenoic	trace
16:1	6-*cis*-Hexadecenoic	0.021
16:1	7-*cis*-Hexadecenoic	0.092
16:1	8-*cis*-Hexadecenoic	trace
16:1	9-*cis*-Hexadecenoic	1.46
16:1	10-*cis*-Hexadecenoic	trace
16:1	11-*cis*-Hexadecenoic	0.042

Table 4.5 (*Continued*)

Acid[a,b]	Structure	Wt. %
16:1	12-*cis*-Hexadecenoic	trace
16:1	13-*cis*-Hexadecenoic	0.0099
16:1	5-*trans*-Hexadecenoic	0.0038
16:1	6-*trans*-Hexadecenoic	0.014
16:1	7-*trans*-Hexadecenoic	0.012
16:1	8-*trans*-Hexadecenoic	0.0087
16:1	9-*trans*-Hexadecenoic	0.057
16:1	10-*trans*-Hexadecenoic	0.0013
16:1	11-*trans*-Hexadecenoic	0.0078
16:1	12-*trans*-Hexadecenoic	0.022
16:1	13-*trans*-Hexadecenoic	0.017
16:1	14-*trans*-Hexadecenoic	0.0078
17:1	6-*cis*-Heptadecenoic	0.0092
17:1	7-*cis*-Heptadecenoic	0.0057
17:1	8-*cis*-Heptadecenoic	0.054
17:1	9-*cis*-Heptadecenoic	0.19
17:1	10-*cis*-Heptadecenoic	trace
17:1	11-*cis*-Heptadecenoic	0.0078
17:1	12-*cis*-Heptadecenoic	trace
17:1	*trans*-Heptadecenoic	0.018
18:1	7-*cis*-Octadecenoic	trace
18:1	8-*cis*-Octadecenoic	0.45
18:1	9-*cis*-Octadecenoic	25.5
18:1	10-*cis*-Octadecenoic	trace
18:1	11-*cis*-Octadecenoic	0.67
18:1	6-*trans*-Octadecenoic	0.030
18:1	7-*trans*-Octadecenoic	0.024
18:1	8-*trans*-Octadecenoic	0.097
18:1	9-*trans*-Octadecenoic	0.31
18:1	10-*trans*-Octadecenoic	0.32
18:1	11-*trans*-Octadecenoic	1.08
18:1	12-*trans*-Octadecenoic	0.12
18:1	13-*trans*-Octadecenoic	0.32
18:1	14-*trans*-Octadecenoic	0.27
18:1	15-*trans*-Octadecenoic	0.21
18:1	16-*trans*-Octadecenoic	0.23
19:1	—	0.06
20:1	—	0.22
21:1	—	0.02
22:1	—	0.03
23:1	—	0.03
24:1	—	0.01
25:1	—	0.0008
26:1	—	0.0008

a Acid designated by number of carbon atoms, colon, number of double bonds.
b Data for C_{10}, three C_{12}, 13-*trans*-tetradecenoic, C_{15}, and C_{18-26} acids taken from reference 122. Data for C_{25} and C_{26} acids taken from reference 144. All other data taken from reference 121.

Table 4.6

POLYENOIC FATTY ACIDS OF BOVINE MILKFAT

Acid [a]	Structure	Ref.	Wt. %	Ref.
14:2	—	144	0.04	144
16:2	—	144	0.02	144
18:2	*cis,cis*-9,12-acid	280	2.11 [b]	122,280
18:2	*cis,cis*-9,15-acid	156		156
18:2	*cis,cis*-10,15-acid	156		156
18:2	*cis,cis*-11,15-acid	156	0.02	156
18:2	*cis,cis*-8,15 and/or 8,12-acid	156		156
18:2	*cis,cis*-7,15 and/or 7,12-acid	156		156
18:2	*cis,cis*-6,15 and/or 6,12-acid	156		156
18:2	*cis,trans* (or *trans,cis*)-11,16 and/or 11,15-acid	357		
18:2	*cis,trans* (or *trans,cis*)-10,16 and/or 10,15-acid	357		
18:2	*cis,trans* (or *trans,cis*)-9,15 and/or 9,16-acid	357		
18:2	*cis,trans* (or *trans,cis*)-8,16 and/or 8,15 and/or 8,12 acid	357[c]	0.44[b]	122,280
18:2	*trans,trans*-12,16-acid	357		
18:2	*trans,trans*-11,16 and/or 11,15-acid	357		
18:2	*trans,trans*-10,16 and/or 10,15-acid	357		
18:2	*trans,trans*-9,16 and/or 9,15 and/or 9,13-acid	357		
18:2	conjugated	122,280	0.28 [b]	122,280
18:3	*cis,cis,cis*-9,12,15-acid	280	0.38[b]	122,280
18:4	—	144	0.10	144
20:2	—	122	0.05	122
20:3	—	122	0.11	122
20:4	*cis,cis,cis,cis*-acid	122,299	0.14	122
20:5	*cis,cis,cis,cis,cis*-acid	122,299	0.04	122
22:2	—	122	0.01	122
22:3	—	122	0.02	122
22:4	*cis,cis,cis,cis*-acid	122,299	0.05	122
22:5	*cis,cis,cis,cis,cis*-acid	122,299	0.06	122
24:2	—	144	0.02	144
26:2	—	144	0.0004	144

[a] Acid designated by number of carbon atoms, colon, number of double bonds.
[b] Calculated roughly from data in references 122 and 280.
[c] Calculated roughly from data in this table and references 122 and 280.

on degree of unsaturation. These are GS_3 (trisaturated), GS_2U (disaturated, monounsaturated, GSU_2 (monosaturated, diunsaturated), and GU_3 (triunsaturated). Hilditch generalized the experimental data in a theory of "even" distribution which in essence stated that an acid tends to have as wide a distribution as possible among the triglycerides. A strict application of this theory, which Hilditch

later said should not be attempted,[125] would require any acid to represent more than 33 mole% of the total acids to occur twice in a triglyceride, and more than 67 mole % to form a simple triglyceride. Sommer,[322] vigorously opposing this theory, pointed out that while the monoacid theory implies a chemical incompatibility of unlike fatty acid molecules, the theory of "even" distribution implies the converse, or an incompatibility of like fatty acid molecules. Hilditch later emphasized[125] that he never intended this rule to be more than an expression of the major tendency. More precisely, in most natural fats the content of glycerides containing one group of a particular acid reaches a maximum when that acid is exactly one-third of the total acids and, likewise, the content of glycerides containing two groups of the acid reaches a maximum when the acid is exactly two-thirds of the total acids. At these maxima about 85 to 90% of the whole fat consists of glycerides with, respectively, one or two groups of the particular acid. Glycerides containing one group of a particular acid persist, in decreasing amounts, until that acid forms about 75% of the total acids, and those containing two groups of the acid start appearing when the acid forms 20 to 25% of the total acids. Simple triglycerides start to appear when the acid forms 60% or more of the total acids.

A third theory, that of random distribution, has been defended by Sommer[322] and by many others. According to this theory, distribution of fatty acids among the glycerides is determined solely by chance and, therefore, follows the law of probability. The total number of triglycerides, including both positional and optical isomers, present in a fat is equal to the third power of the number of fatty acids present. This figure can be subdivided in such a manner as to show the different types of triglycerides included in it, thus:

(1) $G(AAA) = X$
(2) $G(AAB) = 2X(X - 1)$
(3) $G(ABA) = X(X - 1)$
(4) $G(ABC) = X(X - 1)(X - 2)$

in which, as before, X is the number of fatty acids present. The formula in (4) includes the positional isomers G(ACB) and G(CAB). The molecules represented in (2) and (4) are asymmetric and, of the number of different glycerides calculated for each of these types, one-half consists of optical isomers of the other half. X^3 represents the total number of different glycerides if both optical forms of asymmetric molecules are present; $(X^3 + X^2)/2$ represents the number of glycerides if only one optical form of each asymmetric molecule is present. Earlier calculations (This Book, 1965 edition) were made using a figure of 60 fatty acids. On this earlier basis, the 60 fatty acids would be distributed among 216,000 different glycerides including both forms of optical

isomers, or 124,800 different glycerides including but one form. The random theory also permits calculation of the quantitative occurrence of the various glycerides. In the following equations a, b, and c represent, respectively, the mole fractions of acids A, B, and C. By the use of these formulas the mole fraction of any glyceride can thus be calculated. This figure multiplied by 100 gives the mole percent of the glyceride in the fat.

$$G(AAA) = a^3$$

$$\left.\begin{array}{l} G(AAB) = 2a^2b \\ G(ABA) = a^2b \end{array}\right\} = 3a^2b$$

$$\left.\begin{array}{l} G(ABC) = 2abc \\ G(ACB) = 2abc \\ G(CAB) = 2abc \end{array}\right\} = 6abc$$

Doerschuk and Daubert[68] have shown that the glycerides of corn oil do not fit the pattern of either an even or a random distribution of the fatty acids. The partial random theory of these investigators permits an acid present in less than 33 mole % to appear twice in some molecules. It also permits an acid present in 33 to 67 mole % to be absent from some molecules.

Kartha[162] analyzed for the GS_3, GS_2U, GSU_2, and the GU_3 content of 27 natural fats and studied the analyses of 46 natural fats published by others. He concluded that each natural fat has a limit, which cannot be exceeded, for trisaturated glycerides and that its glyceride composition corresponds to that calculated from a random deposition of fatty acids until this limit is reached. Any excess saturated acids are utilized in the random formation of GS_2U and GSU_2 at the expense of GSU_2 and GU_3, respectively. Hilditch[125] has criticized these conclusions. Without obtaining evidence for an even distribution, Quimby *et al.*[268] have nevertheless concluded that the fatty acids are nonrandomly distributed in animal fats. They concluded that lard is composed largely of 2-palmityl glycerides and tallows probably of 1-palmityl glycerides. Vander Wal[346] and Coleman and Fulton[51] developed the 1, 3-random-2-random theory to account for the known deviations from random theory. Jensen and Sampugna[153] have discussed the applicability of the random and 1, 3-random-2-random theories to milkfat. Thus it appears that in many instances—perhaps in all—a completely random distribution of the fatty acids has been modified biologically.

It has been shown that the fatty acids of synthetic fats are randomly distributed. Similarly, if a nonrandomly distributed natural fat is heated with a catalyst, such as sodium methoxide, the fat is resynthesized by an interesterification process into a new material

in which the fatty acids are randomly distributed among its glycerides. Norris and Mattil[242] have used this process as the basis of a test for the presence or absence of random distribution in natural fats and oils. If the characteristics of the fat before and after treatment are identical, it is presumed that the natural fat follows a pattern of random distribution. If the characteristics differ, the original distribution must have been nonrandom. Interesterification of butterfat has been found to change its refractive index,[25] its melting point,[355] and other physical characteristics. It thus appears that the distribution of fatty acids in the glycerides of milkfat is nonrandom.

While the earlier studies of glyceride structure concerned the intermolecular distribution of fatty acids, that of Quimby *et al.* pertained to their intramolecular distribution. Their conclusions were based on data obtained by thermal and x-ray diffraction studies. An additional tool for the study of glyceride structure has been provided by the discovery that pancreatic lipase initially catalyzes the hydrolysis of only primary ester linkages.[217,282,337,338] This lipase has been used in studies of the positional distribution of fatty acids in cow's milk glycerides. The results show considerable conflict of detail, but support the view that at least some of the fatty acids are nonrandomly distributed.

Kumar and his associates[187,189] found that butyric acid is preponderantly in the α-positions. Entressangles *et al.*,[77] on the basis of their findings that pancreatic lipase hydrolyzes butyric acid linkages more rapidly than those of long-chain acids, questioned the validity of results obtained by applying this method to milkfat. Jack *et al.*[145] studied the method and felt that it could be validly applied to milkfat by using conditions which minimize the preferential hydrolysis of butyric acid linkages. They, like Kumar's group, found a large part of the butyric acid in the α-positions.

Several groups[14,222,256] have found a greater than random percentage of saturated acids in the ß-position of cow's milkfat. In this respect these results resemble those found for hog fat and contrast with those found for beef fat,[268] other animal and vegetable fats,[218] and seed fats.[219] Jack *et al.*,[145] however, found a less than random percentage of the saturated acids in the ß-position; palmitic acid, in contrast to the group as a whole, was preferentially located in this position.

Jensen *et al.*[155] found that pancreatic lipase shows no preference for the butyric acid linkage as such. For example, using short digestion periods, it released butyric and palmitic acids in equimolar quantities from glyceryl 1-palmitate-2, 3-dibutyrate. However, this enzyme does discriminate, with respect to the speed with which acids are released from the α-position, between molecular species. These investigators felt, accordingly, that results obtained on milk triglycerides should

be viewed with reservation; those obtained with short digestion periods yield information essentially about the more rapidly hydrolyzed triglycerides; those obtained with prolonged digestion are vitiated by acyl migration, which obscures positional specificity.

A number of others[31,49,132,154,155,197,317] have confirmed the preferential location of butyric acid in the terminal positions of glycerol. In each instance, however, it was either not possible to demonstrate to the contrary, or else positive evidence was obtained for placement of part of the butyric acid in the 2-position.

Caproic acid, also, has been found in the terminal positions in greater than random proportions in milkfat[31,132] and in the milkfat fraction soluble in pentane at −60°C.[317]

Both stearic and oleic acids were found in the terminal positions in greater than random proportions in milkfat[31,132,197] and in various fractions obtained by fractionally crystallizing milkfat.[197]

Myristic acid was found in the 2-position in greater than random proportions in milkfat,[31,64,132,155,197] in various milkfat fractions obtained by crystallization,[197] by silicic acid fractionation,[64] and in the fraction soluble in pentane at -60°C.[317]

Palmitic acid was found in the 2-position in greater than random concentrations in milkfat,[31,132,155,197] in the fraction soluble in pentane at -60°C,[317] and in various fractionally crystallized samples.[197] In one study,[64] palmitic acid was distributed almost randomly between the 2-position and the terminal positions. This fat was fractionated on silicic acid into a series of samples in which the triglycerides varied from high to low molecular weights. In the high molecular-weight samples the palmitic acid was more than random in the 2-position; in the low molecular weight samples it was less than random in the 2-position, its distribution in the 2-position decreasing continuously from high to low in the samples of intermediate molecular weight.

The distribution of fatty acids in the terminal positions has been studied further by a stereospecific analysis[37] which discriminates between positions 1 and 3 of *sn*-glycerol. In this analysis the triglycerides are partially hydrolyzed with pancreatic lipase and the resultant diglycerides isolated chromatographically. The diglycerides, consisting of a mixture of 1, 2-diacyl-*sn*-glycerol and 2, 3-diacyl-*sn*-glycerol, are treated with phenyldichlorophosphate, which reacts with the free hydroxyl groups to form phosphorylphenols. The mixture is reacted with phospholipase A, which can attack only the 1,2-diacyl-*sn*-glycero-3-phosphorylphenol to liberate the fatty acids from the 2-position. The resultant mixture thus contains unchanged 2, 3-diacylphospholipids, lysophospholipids with acids in the 1-position, and free fatty acids. After separation, analysis of these fractions and the

original triglycerides gives sufficient information for designating the distribution of the acids among the three positions of *sn*-glycerol.

Pitas *et al.*[263] have stereospecifically analyzed milkfat. Breckenridge and Kuksis[32,33] have stereospecifically analyzed two fractions obtained by molecular distillation of butterfat. The so-called short-chain triglyceride fraction contained triglycerides with 34 to 44 acyl carbon atoms per molecule and most of the butyric acid in milkfat. The other fraction—the long-chain triglyceride fraction—contained triglycerides with 36 to 54 acyl carbon atoms per molecule. The fatty-acid composition of each fraction is shown in Table 4.7. The results of these stereospecific analyses have been assembled in Table 4.8.

It is obvious that, in general, the fatty acids in the terminal positions are not divided equally between the 1- and 3-positions. Of all the fatty acids, the greatest discrimination is shown in the preferential placement of butyric acid in the 3-position of *sn*-glycerol.

Stull and Brown[334] made a comparative study of the fatty acids in the milkfats of some common dairy breeds. Composite samples from each of three herds (Guernsey, Holstein-Friesian, and Jersey) were taken monthly for a period of two years while the cows were on a uniform diet at a constant energy level according to production. The Holstein-Friesian group differed significantly from the other two with

Table 4.7

FATTY-ACID COMPOSITION OF SHORT- AND LONG-CHAIN TRIGLYCERIDE FRACTIONS [a]

	Mole %	
Fatty Acid [b]	Short-Chain	Long-Chain
4:0	18.3	1.5
6:0	7.5	2.2
8:0	2.0	1.6
10:0	3.5	2.9
12:0	3.1	3.5
14:0	11.0	11.4
14:1	1.0	trace
15:0	1.8	3.8
16:0	27.8	28.2
16:1	1.6	3.0
17:0	1.3	0.9
18:0	6.7	13.1
18:1	13.0	26.1
18:2	1.0	1.2

a Data from reference 33.
b Designated by number of carbon atoms:number of double bonds.

Table 4.8

POSITIONAL DISTRIBUTION OF FATTY ACIDS IN TRIGLYCERIDES [a]

Fatty Acid [b]	1-Position			2-Position			3-Position		
	Total Fat [c]	Short-chain [d]	Long-chain [e]	Total Fat [c]	Short-chain [d]	Long-chain [e]	Total Fat [c]	Short-chain [d]	Long-chain [e]
4:0	9.8	0.0	0.0	5.6	0.0	0.0	84.6	100	100
6:0	16.3	0.0	0.0	25.8	0.0	0.0	58.0	100	100
8:0	17.2	0.0	trace	42.1	15.0	trace	40.7	85.0	<100
10:0	20.7	8.6	13.8	49.7	41.0	32.2	29.6	50.5	54.0
12:0	24.6	33.3	16.2	47.5	69.9	37.1	28.0	− 3.2	46.7
14:0	27.6	32.8	18.5	53.8	69.1	46.8	18.6	− 1.8	34.7
14:1	—	17.1	trace	—	85.7	trace	—	− 3.6	—
15:0	—	42.6	16.7	—	81.5	32.5	—	−24.1	50.9
16:0	45.5	59.3	44.8	41.7	44.8	45.7	12.8	5.9	9.5
16:1	48.5	39.6	31.1	35.6	64.6	42.2	15.9	− 4.2	26.7
17:0	—	84.6	76.1	—	33.3	38.1	—	−17.9	−14.3
18:0	58.2	73.6	48.1	25.9	17.4	21.6	16.0	9.0	30.3
18:1	41.8	50.8	32.8	27.9	30.3	24.8	30.3	19.0	42.3
18:2	36.7	33.3	27.0	77.9	40.0	44.4	−14.6	26.7	27.8

a Percentage of each acid in designated position of *sn*-glycerol.
b Designated by number of carbon atoms:number of double bonds per molecule.
c From reference 263.
d Calculated from mole % data in reference 32.
e Calculated from mole % data in reference 33.

respect to five acids. The milkfat of this group had smaller proportions of capric and lauric acids and larger proportions of palmitic, palmitoleic, and oleic acids. The magnitude of these differences, while significant, was not considered large enough to affect the gross characteristics of the milkfats.

Effect of Species Variation on Fatty Acids.—The various species in Table 4.9 are grouped so as to reflect the extent to which ingested food is subjected to microbial activity in the digestive tract. This is greatest in the ruminants and decreases in subsequent groups; it is probably at a minimum in carnivores. Odd-carbon acids, branched-chain acids, *trans* unsaturations, and conjugated double bond systems are all formed by microbial activity. Such acids in an animal's milkfat may have been formed by activity in the animal itself, or may have been ingested in the animal's food.

Fatty acids of grass lipids are chiefly C_{16} and C_{18}.[311] The fatty acids of the blood glycerides in a cow were found[205] to be chiefly C_{16} and C_{18}. The dietary fatty acids in a standard ration fed rats (Table 4.9) were chiefly C_{16} and C_{18}. As a basis for discussion, we can consider the group of acids from C_4 to C_{14} inclusive to have resulted primarily from mammary gland synthesis. Taking the average composition of the milkfat of each group in Table 4.9, these mammary gland-synthesized acids form the following percentages of the total acids: ruminants, 24.5; nonruminant herbivore, 22.0; omnivores, 18.8. Many individuals species show a greater similarity to species in other groups than do the group averages. It is apparent that the greatest intragroup differences occur among the omnivores. It is also apparent that individual fatty acids vary more widely between species and groups than do the synthesized acids as a whole. The C_4 and C_6 acids are most abundant in the ruminants and decrease in subsequent groups.

The unusually high polyunsaturated acid content of horse milkfat may be due to the availability of an abundance of dietary polyunsaturated acids which are subjected to only limited microbial hydrogenation. Some interesting studies of variations in milkfat-acid composition might be based on the circumstance that the horse has less microbial activity than ruminants, while most other variables such as diet, climate, freedom of m ovement, etc., could be controlled to be either the same, or to vary independently.

The data of Glass *et al.*[101,102] cover the component acids of the milkfat of 91 species representing 14 orders. Additional species not included in these studies are the Eland antelope (*Taurotragus oryx*),[50] the grey (Atlantic) seal (*Halichoerus grypus*),[1] the collared peccary (*Pecari tajacu*),[333] the kangaroo rat (*Dipodomys merriami*),[333] the bats (*Laptomycteris nivalis* and *Tadarida brasiliensis*),[333] the fin whale

Table 4.9

COMPONENT ACIDS (WEIGHT PERCENT) OF THE MILKFAT OF VARIOUS SPECIES

Group Species	Saturated								Odd Carbon	Branched Chain	Mono-ethenoid	Poly-ethenoid	*Trans*	Conju-gated	Ref.
	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}							
Herbivore															
Ruminant															
Cow[a]	2.8	2.3	1.1	3.0	2.9	9.0	24.0	13.2	3.7	1.5	33.3	3.8	5.	1.1	122
Sheep[b]	4.0	2.1	2.0	6.0	2.8	5.3	17.5	15.6	1.5[i]	—	38.6	5.6	12.1	2.7	95
Goat[c]	3.0	2.5	2.8	10.0	6.0	12.3	27.9	6.0	0.9[l]	—	25.1	3.8	—	—	127
Buffalo[d]	3.0	0.2	1.4	1.7	2.6	9.2	37.7	11.5	1.1[l]	—	30.5	0.9	—	—	6
Nonruminant															
Horse[e]	0.4	0.9	2.6	5.5	5.6	7.0	16.1	2.9	trace[i]	—	29.9	28.8	—	—	127
Omnivore															
Rat[f]	—	—	0.4	8.3	11.5	16.1	27.1	3.0	0.4[i]	—	13.9	9.4	—	0.9[j]	22
Hog[g]	[		1.3		]	1.5	26.9	6.5	<0.8[i]	—	45.0	18.8	—	—	59
Human[h]	[	0.3	]	1.63	6.89	8.5	20.9	7.3	2.7	1.2	39.5	10.1	1.0–4.0[k]	—	141
Carnivore															
Cat				1.0	1.9	7.2	24.6	10.4	2.0	—	42.5	10.5	—	—	102
Dog				0–1.5	trace to 1.0	4.0	27.3	4.4	trace to 0.8	—	48.2	14.5	—	—	102

[a] Ayrshire/Philadelphia, Pa./May/grain-bran-molasses supplement (1 lb/3 lb milk produced) + timothy hay ad libitum, 4-hr pasture daily.
[b] Romney/Palmerston North, New Zealand/Sept. (early spring) 4 weeks postpartum/pasture of ryegrass and white clover.
[c] Norfolk, England/hay, kale, beet, 1–1.5 lb peanut cake, 2-hr pasture.
[d] Murrah/India/wheat bran, grain, peanut cake, grain husk (4.0:2.0:1.5:2.5) 1 lb/2 lb milk + 70 lb green grass + 3 lb straw.
[e] Shire/Cheshire, England/summer pasture.
[f] Wistar rats/Ottawa, Canada/12–15 days post partum/fox cubes with 3% fat (fatty acids:some 14:0, 18:0, 18:3; chiefly 16:0, 18:1, 18:2).
[g] No background information given.
[h] Regular hospital and home food taken ad libitum/11 mothers from 9 to 180 days post partum.
[i] Reference 102. The rat was Norway rat.
[j] Reference 149.
[k] Reference 165.
[l] Reference 88. Expressed in mole %.

(*Balaenoptera physalus*),[2] the African elephant (*Loxodonta africana*),[223] the baboons (*Papio anubis, P. cynocephalus,* and *P. papio*),[42] the rabbit (Comune Migliorata breed),[281] and the hooded seal (*Cystophora crista*).[148]

The milkfat of the Eland antelope contrasted to that of other ruminants by having only 0.9% butyric acid and 0.5% caproic acid. The milkfat of the African elephant had the unusually high caprice acid level of 64.5%. The milkfat of the baboon was unusual in containing 37% linoleic acid; this was attributed to the high level of corn oil in its diet.

Freeman *et al.*[88] examined the milkfats of the cow, sheep, Indian buffalo, goat, and human. They found that butyric and caproic acids were predominantly in the terminal positions of glycerol in the milkfat of all these species. Myristic and pentadecanoic acids were preferentially in the 2-position and stearic acid in the terminal positions of all fats. Human milkfat was notable in having a higher proportion of palmitic acid in the 2-position and of stearic acid and oleic acid in the terminal positions than did the milkfat of any of the ruminants.

Dimick *et al.*[64] found that in both cow's and goats' milkfat palmitic acid was placed preferentially in the 2-position in the high molecular-weight triglycerides and preferentially in the terminal positions in the low molecular-weight triglycerides.

Duncan and Garton[70] examined the milkfat of the cow and found palmitic acid preferentially in the 2-position and stearic, oleic, and linoleic acids preferentially in the 1,3-positions.

Breckenridge *et al.*[34] found that the saturated triglycerides of human milkfat consisted chiefly of *sn*-glycerol-1-stearate-2-palmitate esterified at the 3-position with C_{14} to C_{18} fatty acids.

Marai *et al.*[212] stereospecifically analyzed goat and sheep milkfats after separation into short-chain (34 to 44 acyl carbon atoms) and long-chain (40 to 54 acyl carbon atoms) fractions by means of silicic acid chromatography. Each fraction formed about 50% of the total fat. Of the 20 to 23 mole% of C_4 to C_8 fatty acids present in the short-chain fraction, at least 95% were in the 3-position of sn-glycerol in each species. The distribution of the other acids showed a less marked specificity for either the 1- or the 2-position.

The specific placement of the fatty acids could best be accounted for by assuming a common pool of long-chain 1,2-diglycerides which served as precursors of the bulk of both short- and long-chain triglycerides during milkfat synthesis. This concept is in agreement with the results of Kinsella and McCarthy,[174] which suggested that the milkfat triglycerides were produced by the action of 1,2-diglycerides with endogenously synthesized fatty acids. The mechanism of bio-

synthesis of the 1,2-diglycerides could not be inferred from these results. It is noted in this connection that myristic acid, an endogenously synthesized acid, was found preferentially in the 2-position in the milk fat of both these species as well as in that of the cow.[32,33]

Effect of Composition of Diet on Fatty Acids.—Kaufmann *et al.*[165] found that the *trans* unsaturated fatty acids in human milkfat vary considerably with the nature of the diet. *Trans* acids could no longer be detected after 9 to 11 days of a *trans* acid-free diet in milkfat containing 2% *trans* acids on the fifth day of the diet. In experiments with five cows the milkfat produced from grass in September contained 6.3 to 9.0% *trans* acids, but in winter when beet leaves, beet, hay, and concentrates were fed the content decreased to 0 to 1.7%.

Hilditch[124] has summarized the results of a number of investigations in which specific oils have been added to the diet of cows. Other studies have concerned the effect of added oils on the composition of the milkfat of buffaloes[6] and of rats.[22] The daily inclusion in the diet of 7 lb coconut oil cake, rich in lauric and myristic acids, almost doubled these acids in cow's milkfat. Buffaloes' milkfat was affected in a similar but less pronounced fashion. A variety of oils rich in linoleic acid or linolenic acid has failed to appreciably increase the content of either of these acids in the milkfat of cows or buffaloes.

Rapeseed oil contains about 15% linoleic acid and as much as 50% erucic (13-docosenoic) acid. Addition to the diet of 4 oz daily resulted in the incorporation of 3.7% erucic acid and no detectable linoleic acid into cow's milkfat. In contrast, rats, fed a diet of fox cubes supplemented with 20% rapeseed oil, produced milk with a substantial transfer of acids from the oil into the milkfat.[22] The following figures designate, in order, the acid, its percentage content before, and that after the addition of rapeseed oil to the rats' diet: 18:1, 14—35; 18:2, 9—21; 18:3, 0.5—5; 20:1, 0—5; and 22:1, 0—7. When their diet was supplemented with 20% corn oil rich in linoleic acid, rats produced milkfat in which the linoleic acid content was raised from 9 to 40.5%.

The following percentage composition of cod-liver oil unsaturated acids has been reported: 16:1, 10—20; 18:1 + 18:2, 25—31; 20:3, 25—32; 22:3 + 22:4, 10—20. When 4 oz daily was added to cow's diet the fat content of the milk was reduced, the lower fatty acids were reduced to about half their normal proportions, oleic acid was proportionately increased, and the C_{20} and C_{22} unsaturated acids were greatly increased from a normal value of about 1% to about 5%.

A linseed oil supplement, increased from 260 gm per day to 610 gm per day over a 30-day period, decreased the percentage of C_4 to C_{16} acids and increased that of stearic, oleic, linoleic, and linolenic acids in cow's milkfat.[279]

Kuzdzal[194] established that the levels of conjugated dienes in cow's milkfat are directly related to the levels of dietary linolenic acid.

The addition of 15% safflower oil (containing 81% linoleic acid and no palmitoleic acid) to the diet of cows depressed the production of milkfat and changed its composition.[254] The percentage of all saturated acids was decreased and that of unsaturated acids, as a group, nearly doubled, with individual acids (designated as chain length:number of unsaturations) as follows: 18:1 from 27.4 to 46.6%; 18.2 from 1.9 to 2.9%; 18:3 from 1.1 to 2.9%; 16:1 (absent in oil) from 1.7 to 2.8%.

Many of these findings can be explained on the basis of the extensive hydrogenation of unsaturated acids known to take place in the rumens of ruminants. The unusual effects of cod-liver oil supplements are in accord with the hypothesis that the presence of this oil can alter normal ruminal microbial activity. The substantial incorporation of C_{20} and C_{22} polyunsaturated acids suggests less active hydrogenation by ruminal microbes, while the lower content of volatile acids in the milkfat suggests an interference with the normal production of volatile acids in the rumen.

Shaw and Ensor[304] found that when cows were fed a normal diet supplemented with 300 ml daily of cod-liver oil, oleic acid, or linoleic acid, the ratio of propionic to acetic acid in the rumen was greatly increased. The absolute amount of acetic acid was only slightly decreased by the cod-liver oil and linoleic acid supplements and was actually increased over 75% by the oleic acid supplement. In each instance, however, the amount of propionic acid was greatly increased. The volatile fatty acid components of a cow's milkfat were substantially reduced by the inclusion of this same oleic acid supplement. The reduction was greatest in butyric and caproic acids, which were reduced to about one-half the amounts present when the diet did not include an oil supplement. In each of these instances there was also a reduction in the percentage of milkfat in the milk.

Similar changes in fat content, fat composition, and ruminal volatile acids have been found when cows are fed special diets composed chiefly of cooked concentrates supplemented with small amounts of ground and pelleted hay.[47,76,304] The fineness to which hay is ground can in itself affect the proportions of fatty acids in milkfat.[319] Smith *et al.*[315] have reported on the effect of a variety of hays on some of the major fatty acids in milkfat.

Virtanen[351] has described "zero" milk from cows fed on synthetic, protein-free diets in which all the nitrogen is supplied by urea and ammonium salts. Although the total blood lipids are less than one-third the level in cows on normal diets, "zero" milk contains at least as much fat as does normal milk. The fatty-acid composition of this fat,

however, is quite distinctive. From Table 4.10 it is clear that "zero" milkfat has unusually high levels of lauric, palmitic, and palmitoleic acids and unusually low levels of stearic and oleic acids. The levels of branched acids are also distinctly higher in "zero" milkfat.

These milk samples were taken from cows which had undergone a period of adaptation during which the microbial population of the rumen became adapted to the synthesis of the essential amino acids. After adaptation, protozoa had disappeared from the rumen while the number of bacteria had increased enormously to about 50 times that of cows on normal diets.

Table 4.10

FATTY ACID COMPOSITION OF MILKFAT FROM COWS ON NORMAL AND PROTEIN-FREE FEEDS[a]

	Protein-free Feed			Normal Feed
	Cow 1[c]	Cow 2[d]	Cow 2[e]	Herd
Fatty acid[b]	% of Total Fatty Acids			
4:0	2.58	2.63	3.05	3.05
5:0	0.12	0.13	0.05	< 0.02
6:0	1.88	1.90	1.75	1.76
7:0	0.14	0.12	0.05	< 0.02
8:0	1.30	1.08	1.12	1.02
9:0	0.18	0.12	0.06	< 0.02
10:0	3.63	2.38	2.73	2.13
10:1	0.54	0.44	0.40	0.25
11:0	0.30	0.22	0.14	< 0.02
12:0	5.41	3.64	3.74	2.65
12:1	0.20	0.19	0.18	0.08
13:0 br	0.20	0.15	0.17	0.03
13:0	0.55	0.51	0.26	0.04
14:0 br	0.18	0.32	0.21	0.09
14:0	11.99	10.17	10.95	987
14:1	1.63	2.63	1.95	1.01
15:0 br	0.80	0.52	0.82	0.50
15:0	4.09	4.88	2.70	0.90
16:0 br	0.11	0.48	0.33	0.19
16:0	40.29	48.16	38.90	28.41
16:1	3.54	4.29	4.22	2.03
17:0 br	1.08	0.94	0.96	0.58
17:0	1.21	1.57	0.90	0.46
17:1	0.77	1.13	0.77	0.39
18:0	1.93	0.98	2.58	12.29
18:1	13.18	8.35	19.14	29.37
18:2	1.48	1.40	1.15	1.79
18:3	0.68	0.67	0.74	1.11

[a] From reference 351.
[b] Designated by number of carbon atoms:number of double bonds; br is branched.
[c] Daily diet included 36.6 gm olive oil, 18.4 gm corn oil, and 18.6 gm linseed oil.
[d] Daily diet included 0.0 gm olive oil, 18.4 gm corn oil, and 18.6 gm linseed oil.
[e] Daily diet included 36.6 gm olive oil, 46.0 gm corn oil, and 46.5 gm linseed oil. Different lactation cycle from preceding.

Changes in ruminal volatile acids are partly due to changes in the proportions of various species of ruminal microbes, but may also be partly due to an altered ruminal environment affecting the proportions of the end products formed by individual species.[40] The reasons for these effects have not been completely elucidated. Certain factors, such as the concentration of iron, the presence of oxidizing salts, and the pH are known to affect the proportions of end products.

Leffel[201] incubated whole rumen fluids with various concentrations of C^{14}-labeled glucose. When the glucose was added in trace amounts its label was distributed uniformly among the volatile fatty acids. When the glucose was added in large amounts (20 to 40 μ-moles/ml of incubate) the resultant propionic acid contained from 40 to 60% of the activity present in the volatile fatty acids. This was true whether the rumen fluids were obtained from animals on a high roughage, acetate-producing diet, or on a high concentrate, propionate-producing diet. From these results it was concluded that the concentration of glucose at any particular time influences the volatile fatty acids formed, and that the high production of propionic acid observed in animals on high concentrate diets can be accounted for, at least in part, by the rapid rate at which concentrates are fermented.

Van Soest has reviewed[347] theories attempting to explain how these special diets depress the milk fat concentration. Several of these theories lead to situations resulting in an eventual shortage of β-hydroxybutyric acid, the acid which Avena and Kumar[15] have shown to be especially important in the production of butyric and caproic acids within the mammary gland. It was noted just above that these are the acids that are especially depressed by a dietary oleic acid supplement. Higher levels of propionic acid lead to the formation of more glucose by the animal's metabolism. High levels of glucose are known to depress the oxidation of fat by the liver. Thus, less β-hydroxybutyric acid is formed and less of this important material is available to the mammary gland for synthesis of the lower fatty acids.

Plane of Nutrition.—The plane of nutrition can have a pronounced effect on the composition of milkfat. In Table 4.11 a comparison is made of milkfats produced just before and during a period in which food was withheld completely from the animals. Cow number 1 seemed to present a case of simple inanition. The milkfat yield, on the 11th and 12th days of fasting, was 40% of the original value. With cow number 2 the data are complicated by the development, on the second day of fasting, of severe symptoms of "milk fever" which, however, responded rapidly to treatment. During this complication the secretion of milk and of milkfat almost ceased. By the end of the experiment the fat production had risen to 20% of its original value. These data

show that fasting produces effects which are similar to, but more pronounced than those of the special diets described above. All the lower acids, butyric through myristic, i.e., the group previously referred to as arising chiefly from mammary gland synthesis, are greatly reduced in percentage.

Effect of Miscellaneous Factors on Fatty Acids.—Analyses of English milkfats have shown typical changes when the animals go from winter stall feeding to pasture grazing. These changes normally include a sudden increase in the iodine value and, as shown in Table 4.12, an increase in the oleic acid content of the fat. The stall-fed animals referred to in this table had received the usual winter diet of that period consisting largely of hay, roots, and farm-grown concentrates. The silage-fed group had been receiving only hay and silage. The other two groups had been feeding on fresh grass of early and late summer. From the fact that the acid composition of the hay and silage-fed group was similar to that of typically winter-fed animals, Hilditch and Jasperson[126] concluded that the observed differences between winter and summer milkfats cannot be directly related to

Table 4.11

EFFECT OF FASTING ON COMPOSITION (WEIGHT PERCENT) OF MILKFAT ACIDS[a]

Acid	Cow No. 1		Cow No. 2
	Before Fasting	During Fasting[b]	During Fasting[c]
Butyric	3.5	1.2	2.7
Caproic	0.6	—	0.1
Caprylic	1.0	0.1	0.1
Capric	1.8	0.2	1.0
Lauric	2.5	0.1	0.6
Myristic	11.9	2.8	3.8
Palmitic	23.5	20.0	22.1
Stearic	11.6	14.3	9.9
Arachidic	1.1	0.9	0.9
9-Decenoic	0.2	—	0.2[d]
9-Dodecenoic	0.2	—	0.2[d]
9-Tetradecenoic	0.9	0.4[d]	0.4[d]
9-Hexadecenoic	3.2	1.4	2.0
Oleic	35.9	52.8	51.7
Octadecadienoic	1.2	2.5	0.8
C_{20} unsaturated	0.8	3.3	3.5

[a] Data from reference 314.
[b] Sample from mixed fat secreted on 11th and 12th days of fasting.
[c] Sample from mixed fat secreted on 7th to 12th days of fasting.
[d] Considered by Smith and Dastur to be somewhat higher than true values.

Table 4.12

SEASONAL VARIATION (WEIGHT PERCENT) OF COMPONENT ACIDS OF MILKFAT

Acid	Stall-Fed Winter[a]	Silage-Fed Winter[a]	June Pasture[b]	August Pasture[b]
Butyric	3.0	3.6	3.7	3.5
Caproic	1.4	2.0	1.7	1.9
Caprylic	1.5	0.5	1.0	0.7
Capric	2.7	2.3	1.9	2.1
Lauric	3.7	2.5	2.8	1.9
Myristic	12.1	11.1	8.1	7.9
Palmitic	25.3	29.0	25.9	25.8
Stearic	9.2	9.2	11.2	12.7
As Arachidic	1.3	2.4	1.2	1.5
9-Decenoic	0.3	0.1	0.1	0.1
9-Dodecenoic	0.4	0.1	0.2	0.2
9-Tetradecenoic	1.6	0.9	0.6	0.6
9-Hexadecenoic	4.0	4.6	3.4	2.4
Oleic	29.6	26.7	32.8	34.0
As Octadecadienoic	3.6	3.6	3.7	3.7
As C_{20-22} unsaturated	0.3	1.4	1.7	1.0

[a] Data from reference 129.
[b] Data from reference 126.

dietary changes. They postulated that these seasonal differences may result from differences in freedom of movement and exercise between stall-fed and pasture-grazing animals.

Similar changes in fatty-acid composition have been observed in other parts of the northern hemisphere. Jensen *et al.*[152] found that Connecticut milkfat was richer in oleic and stearic acids and poorer in myristic acid in summer than in winter. Patton *et al.*[260] compared the acid composition of late winter (March) and early summer (June) milkfats. They found the following percentage increases in the summer fat: 18:0, 4.2; *cis*-18:1, 4.4; *trans*-18:1, 3.9; 18:2 + 3, 0.4. These increases were mainly at the expense of palmitic acid (9.0% loss) and myristic acid (2.9% loss).

Milkfat samples from both southern and central Alberta were examined by Wood and Haab.[360] The iodine value reached a maximum in May in the southern fat and in September in the northern fat. It reached minimum values in November to December in both regions. Oleic acid reached maximum values in May and minimum values in November in both regions. The nonconjugated polyunsaturated acids did not vary geographically. The dienoic fraction did not vary seasonally. The trienoic fraction reached a maximum in July. The conjugated polyunsatuated acids had maximum values during the grazing

season, May to September, and minimum values during the stall-feeding period.

The iodine value of butterfat in France[196] was found to be higher throughout the year in evening milk (36.9 average) than in morning milk (34.2 average). The nonconjugated diene and tetraene acids were relatively constant throughout the year. They averaged 1.1 and 0.34 gm/100 gm fat, respectively. The conjugated diene acids increased during the grazing period, late April to November. They averaged, in the winter, 0.5 and in the summer, 1.6 gm/100 gm fat. Nonconjugated triene, i.e., linolenic acid, averaged in the summer 0.64, and in the winter 0.40 gm/100 gm fat. This acid was also slightly higher in evening than in morning milkfats.

The relationships between season of the year and milkfat composition in New Zealand are quite different from those recorded in the northern hemisphere. Hansen and Shorland[112] examined bimonthly samples of milkfat from cows grazing throughout the year on pasture which reaches its maximum lushness within the months of September to January and slowly declines thereafter. The lactation period starts about the middle of July, reaches a peak in November, and ends in May. During the winter the cow's diet is normally supplemented with hay or silage.

The analyses of Hansen and Shorland showed that the iodine values were highest in July and September and dropped sharply to a minimum in November. The greatest changes in fatty-acid composition also occurred in the November samples. The C_{18} unsaturated acids dropped from 29.5 mole % at the beginning of lactation to 23.4 mole % in November. The C_6 to C_{14} acids increased during this same period, from 18.6 to 26.8 mole %. The seasonal changes were interpreted as resulting from changes in the plane of nutrition and the stage of lactation. Later analyses of sheep milkfat[95] from sheep grazing under similar conditions showed that similar fatty-acid compositional changes took place at the same time of year as those in cow's milkfat. Since the stages of lactation were completely different in the two species, it was concluded that the plane of nutrition is the most important factor in producing seasonal changes in milkfat composition.

In French butterfat,[195] 2.3% of the total acids were *trans* in March (in stalls) and 7.0% were *trans* in May (on pasture). Vaccenic was the major *trans* acid. Pentadecenoic acid was mostly *trans*, hexadecenoic acid was 15%, and myristoleic and heptadecenoic acids each less than 15% *trans*. Antilla[11] has reported on the seasonal variation of conjugated, *trans*, and branched acids in Finnish butterfat.

No significant difference was found in the fatty-acid composition of morning and evening milks.[272,335] A number of studies on cow's

milk[3,57,272,335,336] and one on human milk[270] showed significant increases in the short-chain acids from colostrum through at least 8 weeks of lactation. The results of Hosogai[139] were in conflict with these findings.

The effect of high temperatures and humidity on milkfat composition was studied by Richardson *et al.*[273] The experiments were designed to maintain all other conditions constant. It was found that typical changes in fatty-acid composition took place when any combination of high temperature and high relative humidity resulted in a rectal temperature of 103 to 105°F. This situation was marked by a relative decrease in the low-melting acids, short-chain and oleic, and a relative increase in the higher-melting acids, chiefly palmitic and stearic.

Similar effects were found by Moody *et al.*[231] Comparisons were made between one group of cows held two weeks at 15 to 24°C and another group at a constant 32°C and 60% relative humidity. The milkfat of the latter group had depressed levels of 12:0, 14:0, 14:1 + 15:0, 18:1, and 18:2 acids. The lower acids were not determined.

In concluding this section on factors affecting the acid composition of triglycerides, it is noted that apparently unrelated data may sometimes be unified or what seem to be contradictory results resolved if coordinating data are available on the animal's metabolism and that of the rumen flora. Two examples come to mind. In the first, both starvation and high-concentrate diets, representing the two extremes of the plane of nutrition, lower the relative proportion of the C_4 to C_{14} acids in milkfat. Because of the important relationship of β-hydroxybutyric acid to the C_4 and C_6 acids,[15] a shortage of this precursor might adequately explain the preferential depression of these acids in the milkfat of cows on a high concentrate diet. During starvation there is no relative shortage of β-hydroxybutyric acid. However, in addition to a shortage of acetic acid, there appear to be metabolic changes which interfere with the mammary gland's ability to use either of these substrates normally for fatty-acid synthesis.[208]

The second example concerns the varying extent to which polyunsaturated acids have been found to be incorporated into milkfat. It is well known that in normal diets extensive ruminal hydrogenation acts as a barrier to the passage of these acids into milkfat. Since neither these acids nor their parent oils are water-miscible, the extent to which they undergo ruminal hydrogenation must be influenced by the manner in which they are presented to ruminal action. For example, a single massive dose of an oil probably would be less completely hydrogenated than the same amount administered in a number of smaller portions.

Mochrie and Tove[230] have presented data demonstrating the

extremes of this principle. On the one hand, soybean oil was fed as the whole bean so that the oil, by a slow release from the bean, would be subjected to a maximum of microbial action. On the other hand, cottonseed oil was infused into an animal intravenously so that ruminal action would be completely bypassed. Linoleic acid from the soybean feeding increased in the milkfat from 2.1 to 3.2%; that from the cottonseed oil infusion increased to nearly 15%.

Scott *et al* [297,298] have attempted to bypass ruminal hydrogenation in a way that would be practical for producing milk with a softer fat and higher levels of polyunsaturated acids. Particles containing a core of polyunsaturated oil encapsulated with protein were prepared by spray drying a homogenate of sodium caseinate and the desired oil. The particles were then treated with formalin to protect the protein coat from microbial proteolysis. When these protected oils were fed to ruminants they largely escaped hydrogenation in the rumen; the formaldehyde-protein complex was hydrolyzed in the acidic conditions of the abomasum, and the fatty acids were absorbed from the small intestine. The levels of octadecatrienoic acid were raised to from 12 to 22% in the milkfat of cows and to from 21 to 25% in that of goats with encapsulated linseed oil. The levels of octadecadienoic acid in the milkfat of cows were raised to 25% with sunflower oil, 20% with corn oil, 21% with peanut oil, and 35% with safflower oil. Safflower oil also raised the level of this acid to 35% in the milkfat of a goat.

McCarthy[221] has stated that milkfat from a cow on a high-concentrate diet contained the unusually high levels of 8.6% linoleic acid and 2.3% linolenic acid. Both high-concentrate diets and added oils have been shown to affect the formation of volatile fatty acids in the rumen. The extent to which this is associated with metabolic changes that also affect the capacity for hydrogenating fatty acids is not known. It should be considered in relation to the effects of both high-concentrate diets and added oils.

Keto Acid-containing Triglycerides

β-Keto Acid Glycerides.—A homologous series of methyl ketones with an odd number of carbon atoms, C_3 to C_{15} inclusive, has been identified in the unsaponifiable matter of milkfat.[260a] The concentration of these ketones is increased by heating. This circumstance has led to the postulation of glyceride-bound β-keto acids as their precursor.[28] Support for this hypothesis was obtained by the isolation of pyrazolones from butterfat treated with Girard T reagent.[349] Also, synthetically prepared glycerides containing β-keto acids reacted similarly, forming methyl ketones on heating and pyrazolones on treatment with Girard T reagent. Parks *et al.*[249] demonstrated the validity

of this hypothesis by isolating the precursor and identifying it as a triglyceride containing one β-keto acid.

The formation of methyl ketones from precursors is a two-step process: (a) hydrolysis of the ester linkage binding the β-keto acid in the triglyceride, and (b) decarboxylation of the unstable free acid. The hydrolytic step requires water,[199,293,349] and is completely inhibited if the milkfat is adequately dried, as with calcium carbide. Both steps are accelerated at elevated temperatures. Langler and Day[199] obtained maximum ketone formation by heating the fat for 3 hr at 140°C. Obviously, the β-keto ester linkage is much less stable than that of the fatty acids, since complete hydrolysis is obtained under conditions which leave the bulk of the milkfat intact.

Parks *et al.*[249] found that the β-keto acid-containing glycerides represent 0.045% of the milkfat and have fatty-acid components typical of those in milkfat, except for a slightly higher palmitic acid content. The β-keto acid moieties had the following composition, expressed in mole %: C_6, 11.5; C_8, 25.7; C_{10}, 13.8; C_{12}, 11.4; C_{14}, 13.2; C_{16}, 24.4.

The methyl ketone potential of milkfat can vary widely. Parks *et al.*[249] found 0.58 μmoles, Schwartz *et al.*[292a] 0.4 to 1.2 μmoles, and Langler and Day[199] 1.7 μmoles per gm fat.

Dimick and Walker[66] studied the effect of a number of factors on the monocarbonyl potential. As the methyl ketones generated by heating the moist fat were found to compose from 70 to 90% of the monocarbonyls, the conclusions regarding monocarbonyls can, in a general way, be applied to the methyl ketones, and hence to the β-keto acid-containing glycerides.

Samples taken weekly from herd milk over a period of one year showed that the monocarbonyl potential followed a seasonal trend, being higher in the winter (while on barn feeding) and lower in the summer (while on pasture feeding). The monocarbonyl potential correlated, in this respect, with the lactone potential[63] and the levels of short-chain fatty acids.

Studies on three groups of cows, Holstein, Brown Swiss, and Jersey-Guernsey, showed no significant correlation between monocarbonyl potential and breed of cow. This was also true of the lactone potential.[63]

The effect of the stage of lactation on monocarbonyl potential was studied with individual cows throughout a complete lactation period. Both monocarbonyls and methyl ketones (which were assayed separately in this study) increased with the progress of lactation. The lactone potential likewise increased.[63]

Analyses of the milkfat from high fat- and low fat-producing cows showed no significant difference in the monocarbonyl potential.

Ketosis produced low levels of the C_5 to C_{15} methyl ketones.

Other Keto Acid Glycerides.—Vander Ven[348] removed the β-keto acids from milkfat by steam deodorization and then extracted a mixture of other keto acids with Girard T reagent. After reduction of the keto groups to hydroxyl groups, the 4- and 5-hydroxy acids were converted to lactones, which were identified by gas liquid chromatography. The lactones, corresponding to 2.5 ppm in the fat, consisted mainly of δ-octa, δ-deca, and δ-dodecalactones with lesser percentages of γ-deca, γ-undeca, and γ-dodecalactones. From these results the presence of the corresponding 5- and 4-keto acid-containing triglycerides can be inferred.

Keeney *et al.*[170] studied the total keto acids of milkfat and found them to consist chiefly of isomeric ketostearic acids with low percentages of C_{10} to C_{16} saturated keto acids. Unsaturated C_{18} keto acids were also found. The percentage composition of the ketostearic acid fraction was as follows: 8-ketostearic, 1.2; 9-ketostearic, 39.4; 10-ketostearic, 27.5; 11-ketostearic, 10.1; 12-ketostearic, 4.6; and 13-ketostearic acid, 17.3. The levels of keto acid-containing triglycerides in Table 4.1 were calculated from the data obtained in this study.[168] These triglycerides, with the exception of the diglycerides and possibly of the phospholipids, are the most abundant, on a weight basis, of the minor components of milkfat. The keto acid components of these glycerides form about 0.3 to 0.5% of the component acids of milkfat.

Hydroxy Acid-containing Triglycerides

Lactonogenic 4- and 5-Hydroxy Acid Glycerides.—In 1956 Keeney and Patton[171] identified δ-decalactone and in 1960 Tharp and Patton[339] identified δ-dodecalactone in butterfat. The list has been expanded to where these became recognized as members of a homologous series, all the members of which have been identified from C_6 through C_{16}. These, along with a similar series of γ-lactones, have been assembled in Table 4.13. Additional lactones, not members of either of these series, have unsaturations and branchings. They are: 5-hydroxy-9-dodecenoic acid lactone,[364] 4-hydroxy-6-dodecenoic acid lactone,[364] 5-hydroxy-9-tetradecenoic acid lactone (indicated but not proved),[364] 2,3-dimethyl-4-hydroxy-2,4-nonadienoic acid lactone at a level of 0.00005% in butterfat,[28] and *trans*-4-methyl-5-hydroxyhexanoic acid lactone.[137] The last-named lactone was found "free" at a level of 0.00000005% in butterfat. This will be discussed below.

The fact that heating butterfat increases the yield of lactones led to the postulate that the lactones are derived from nonvolatile precursors, namely, triglycerides containing lactonogenic hydroxy acid components. Boldingh and Taylor[28] lent weight to this hypothesis by synthesizing δ-hydroxy acid-containing triglycerides and finding that they

Table 4.13

SATURATED n-ALIPHATIC LACTONES IN BUTTERFAT

Number of Carbon Atoms	δ-Lactones Levels in Butterfat ppm	Ref.	γ-Lactones Levels in Butterfat ppm	Ref.
6	2.0	252	trace	67
7	trace	67	trace	67
8	3.8[a]	65	0.5	175
9	trace	67	0.2	175
10	17.7[a]	65	1.2	158
11	0.7	158	0.5	158
12	28.6[a]	65	1.6	158
13	1.5	158	0.5	158
14	40.7[b]	65	1.4	158
15	6.4	158	1.3	158
16	32.3[b]	65	1.3	158
18	35	362	—	—
19	5[c]	361	—	—

[a] Average values of lactones obtained by steam deodorization.
[b] Average values of lactones obtained by silicic acid chromatography.
[c] δ(?) – C_{19}

did generate lactones on heating. In later work[175,253,362], the lactone precursors in butterfat were concentrated by silicic acid chromatography prior to further study.

Parliment *et al.*[253] concentrated all the lactone precursors in a polar fraction that contained 0.2% of its weight in bound δ-decalactone and 0.8% in bound dodecalactone. Based on chromatographic behavior and molecular weight considerations, they concluded that the precursors are triglycerides containing one esterified hydroxy acid moiety.

Kinsella *et al.*[175] concentrated all the lactone precursors in a polar fraction which represented 0.75% of the butterfat. From this fraction, after heating, they identified most of the saturated lactones known to be derivable from butterfat. After pancreatic lipase hydrolysis of the lactone-precursor fraction, the resultant 2-monoglycerides did not form lactones on heating. From this they concluded that the hydroxy acids are esterified in the α-positions.

Wyatt *et al.*[362] resolved a polar fraction which, from their description, appeared to have a high concentration of lactone precursors. They found that its infrared spectrum strongly suggested that the precursors are hydroxy acid-containing triglycerides.

The level of free lactones in fresh butterfat must be low, since their odor could not be detected in a concentrated percursor fraction.[175] However, during storage of butterfat or dried whole milk, even under

refrigeration, lactones are formed until their presence can be detected by smell and by taste. On the contrary, in sterile concentrated milk[203] the δ-C_{10} and δ-C_{12} lactones decreased during storage at all temperatures studied, while the δ-C_{14} lactone decreased at 4°C and 37°C. Lactone formation is a two-step process: hydrolysis of the glycerol-hydroxy acid ester linkage and lactonization of the free hydroxy acid. The first step requires a low concentration of water for its initiation.[362] It is relatively slow at room temperature or below, usually requiring a period of weeks before the lactones become noticeable. It can be speeded up enormously at higher temperatures. Boldingh and Taylor[28] reported nearly 100% formation of lactone from synthetic triglycerides containing δ-hydroxy decanoic acid when heated at 145°C for about 85 min.

The lactonization step is generally reported as rapid, the expression "spontaneous lactonization" often being encountered. This step does, however, depend on the pH. Under sufficiently alkaline conditions, salts of the hydroxy acids are formed. Kontson *et al.*[182] found that at room temperature a pH well below neutrality was needed for complete and rapid lactonization. Studies of the rate of lactonization in butterfat or in milk at room temperature or lower, if reported, are unknown to this author. Possibly, the anomalous behavior of lactones in sterile concentrated milk involved an equilibration with hydroxy acid salts.

Lactones originating in milkfat are optically active,[27,28] indicating a biological origin. However, they are not optically pure,[350] which suggests that both optical antipodes are formed in milkfat in unequal proportions, by different pathways.

Flavor thresholds of the lactones have been included in a review by Kinsella *et al.*[176] and have been determined and compared with those of other flavor compounds by Siek *et al.*[313] The lactone of 2,3-dimethyl-4-hydroxy-2,4-nonadienoic acid has been described as celery-like,[28] δ-decalactone as coconut-like, and δ-dodecalactone as peach-like. The last two are the most important contributors to the flavor complex arising from the decomposition of lactone precursors in milkfat. These flavors, in themselves, are pleasant. At very low levels they have been considered by some to be important contributors to the desirable flavor of fresh milk and at rather high levels to the desirable flavor imparted by butter to candy and baked products. In this connection it has been claimed in patent applications[345] that lactones impart a buttery flavor to margarine.

High levels of lactones in fresh milk, however, are foreign to its natural flavor and are generally considered a flavor defect. The development of lactones in stored whole milk powder, therefore, has been one factor which has mitigated against its acceptance as a replacement

for fresh milk. Patton was issued a patent[255] for stabilizing the flavor of milkfat against an excessive formation of lactones. The process utilized vacuum steam deodorization, which decomposes the lactone precursors and volatilizes the resultant lactones. Powders with an initial flavor equal to that of fresh whole milk, and with excellent storage qualities, have been prepared from a homogenate of skim milk and steam-deodorized butterfat.[193]

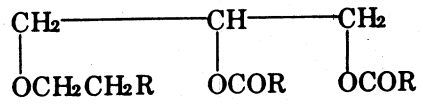
Dimick and Horner[63] examined the effect of several factors on the lactone potential of milkfat. On barn feed, in the winter, butterfat samples averaged 96 ppm lactones (range 59 to 139), while on pasture feed, in the summer, the average was 67 ppm lactones (range 48 to 100). During a 310-day lactation period of one Holstein, the concentration was about 25 to 30 ppm following parturition. It increased to from 170 to 180 ppm at 150 days, and then decreased to final values of about 120 ppm. The effect of breed was examined with groups of Holsteins, Brown Swiss, and Jersey-Guernsey cows. The Holsteins showed a somewhat higher lactone potential than did the other groups. Ketosis lowered the potential markedly. There was a high positive correlation between the lactone potential and the levels of short-chain fatty acids, suggesting a connection in the biosynthesis of the two classes of acids.

"Zero" milk[351] had concentrations of C_6 to C_{10} δ-lactones similar to those in normal milk while the C_{12} and C_{14} δ-lactones were higher than in normal milk. It is not clear whether the reference is to free lactones or to their precursors. However, *trans*-4-methyl-5-hydroxyhexanoic acid lactone[137] was removed by sweeping butterfat warmed to 50°C with carbon dioxide under vacuum. Under these conditions, designed to recover only free lactones, "zero" milk had 20 times the level of this lactone found in normal milk (10 μg vs. 0.5 μg/kg butterfat).

The level of lactones shown in Table 4.13 corresponds to about 0.0167% of the butterfat. With an average molecular weight of 212 for the lactones and 251 for the fatty acids, the lactonogenic hydroxy acid-containing triglycerides have an average molecular weight of 770 and are equivalent to about 0.061% of the butterfat.

Other Hydroxy Acid Glycerides.—Although a number of investigators have reported the presence of nonlactonogenic hydroxy acid triglycerides, there seem to have been no studies of their composition. Schwartz[291] found levels of hydroxy acid triglycerides corresponding to 0.60 to 0.66% of the butterfat. This is in close agreement with the values of 0.61 to 0.78% found in total milk lipids by Timmen.[341] These values, in conjunction with those for lactonogenic triglycerides, indicate that the nonlactonogenic glycerides constitute about 90% of the total hydroxy acid-containing triglycerides.

Neutral Glyceryl Ethers



It can be conjectured that the naturally occurring glyceryl ethers are triglycerides containing two esterified fatty acids. However, investigations of this class of compounds have been made on material isolated from the unsaponifiable fraction of milkfat; hence any fatty acids originally present would have been removed by the preliminary saponification. The presence of the ether linkage on the α-carbon of glycerol has been demonstrated by Davies *et al.*[54] and by Karnovsky and Brumm.[160]

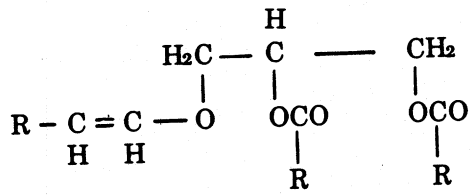
The glyceryl ether content of cow's milkfat has been reported to be 0.0045% by Schogt *et al.*[289] and 0.01% by Hallgren and Larsson.[106] Both groups isolated their material from the unsaponifiable fraction and based their calculations on fatty acid-free compounds. Schogt *et al.* assumed, for their calculations, a palmityl glyceryl ether; the alcohol components found by Hallgren and Larsson are listed just below.

Hallgren and Larsson[106] have investigated the glyceryl ethers of both human and cow's milk. The lipids of human milk contained 0.1% glyceryl ethers and, as stated above, those of cow's milk 0.01%. The monohydric alcohol components of cow's milk glyceryl ethers were found to be palmityl, stearyl, and oleyl in the ratios 1.15:1.00:0.73, respectively. It was felt by these investigators that the composition is more complex than this, the findings being limited by the small amount of available material.

Following are the alcoholic components and their percentage occurrence in the glyceryl ethers of human milk: 16:0—23.9; 16:1—trace; 17 (including both normal and branched)—3.6; 18:0—22.8; 18:1—33.8; 18:2—1.4; 19 (including both normal and branched)—2.4; 20:0—1.6; 20:1—2.3; 22.0—0.7; 22:1—3.4; 24—2.1; unidentified components, 2.0.

As the data of Hallgren and Larsson were obtained from an examination of the total lipids of cream, the possibility that phosphatidic glyceryl ethers were involved is not excluded. However, Schwartz and Weihrauch[291] found the same glyceryl ether level, 0.016 to 0.020% in butterfat, as in the total milk lipids. These levels, based on triglycerides containing two fatty-acid components of average molecular weight, are recorded in Table 4.1. They identified $C_{10,11,12,13,14,15,16,17}$ and $_{18}$ saturated alcohols and indications of both higher- and lower-molecular weight saturated alcohols; 60 unsaturated alcohols were detected, of which only $C_{18:1}$ *and* $C_{18:2}$ were identified with certainty.[291,294]

Neutral Plasmalogens



In this structure, the presence of the ether linkage on the α-carbon of glycerol is indicated by the structural investigations of Eichberg *et al.*[72] on material isolated from the common starfish. The work of Schogt *et al.*[289] on the neutral plasmalogens of milkfat indicated that the major portion of this material likewise had the vinyl ether linkage attached to the α-carbon atom of glycerol.

The aldehydes formed by hydrolysis of the neutral plasmalogens of milkfat have been examined by Schogt *et al.*[290] and by Parks *et al.*[250] Each group found a mixture of normal and branched-chain aldehydes, the branched C_{14} and branched C_{15} being by far the most abundant. Schogt *et al.* found all chain lengths from C_{13} through C_{18}, while Parks *et al.* found all chain lengths from C_9 through C_{18}. The values in Table 4.1 are calculated from these studies.

Diglycerides

The reported levels of diglycerides in fresh milk fat vary considerably: 0.25 to 0.48%,[154a] 1.26 to 1.59%,[341] 4.4 to 6.6%,[30] and 4 to 6 mole %.[220]

The free hydroxyl group is in one of the terminal positions of the diglyceride.[342,343]

Boudreau and deMan[30] reported a fatty-acid composition similar to that of diglycerides formed by partial hydrolysis of butterfat with pancreatic lipase. Their finding of relatively high concentrations of short-chain acids in the diglycerides is unfavorable to a proposal[258] that short-chain acids are esterified in the final step of triglyceride synthesis.

In contrast, Timmen *et al.*[342] have reported a fatty-acid composition which favors this sequence of synthesis. Following is a typical analysis: C_4, < 0.1%; C_6 ≤ 0.1%; C_8, 0.4%; C_{10}, 0.4%; C_{12}, 1.2%; C_{14}, 9.2%; $C_{14:1}$, 1.5%; C_{16}, 36.5%; $C_{16:1}$, 1.8%; C_{18}, 13.9%; $C_{18:1}$, 27.7%; $C_{18:2}$, 2.3%; $C_{18:3}$, 1.2%.

It would appear that these conflicting results are a consequence of the methodologies used in the various studies. The levels of diglycerides recorded in Table 4.1 are those of Timmen and his

associates.[341,342] These values, from mixed herd milks, had no distinct seasonal trends. From individual cows, values within the same range were obtained, with occasional lower values down to 0.91%. In the milk of a single cow, the diglyceride content increased steadily from 0.84% of the fat immediately after parturition to 1.40% after 2 months.

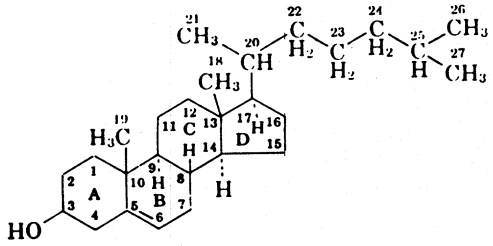
Monoglycerides

The content of monoglycerides, presented in Table 4.1, represents measurements on completely fresh milkfat.[151] Milk taken directly from the animals, without cooling, was treated at once to inactivate lipolytic enzymes.

MISCELLANEOUS NEUTRAL LIPIDS

Steroids

Cholesterol, General Properties.—Cholesterol is insoluble in water, sparingly soluble in cold alcohol or petroleum ether, and soluble in hot alcohol and most other organic solvents. Its solubility in cold ethyl acetate differentiates it from many other lipids. The presence of asymmetric carbon atoms at positions 3, 8, 9, 10, 13, 14, 17, and 20 imparts optical activity to the molecule. Values of −34.3° in chloroform and of both −31.1° and −29.0° in ether have been reported. Cholesterol melts at 148.5°C; it can be sublimed, and distilled under high vacuum.



Cholesterol

The formula of cholesterol, above, shows the system used for designating the various rings and carbon atoms. The use of a solid line for showing the attachment of hydrogen or an alkyl group to a nuclear carbon indicates that the atom or group extends above the general plane of the steroid nuclear ring system, while the use of a dotted line indicates an extension below this plane. Thus, it is seen that in cholesterol the B and C rings and the C and D rings have a *trans* relationship. This is common to all the steroids. Steroids with the configuration at C_3, as shown for cholesterol, are referred to as β-compounds.

Cholesterol appears to be a component of all higher animal cells and is particularly abundant in nervous tissue. A number of suggestions have been made regarding its function in the animal organism. It has been suggested as a protective agent for nervous matter, as controlling cell permeability, as a precursor of the bile acids and some of the hormones, including the sex hormones, and of provitamin D_3. Attempts have been made to correlate the incidence of such disorders as atherosclerosis with a high level of cholesterol in the blood.

For a discussion of its biological functions as well as its biosynthesis and its chemistry the reader is referred to the monograph by Kritchevsky.[185]

In addition to many reactions unique to itself and related steroids, cholesterol shows a number of reactions to be expected from the presence of a carbon-carbon unsaturation and of a secondary alcohol group. A reaction of particular interest, because of its use in the isolation and determination of cholesterol, is that with digitonin to form the molecular addition compound, $C_{56}H_{92}O_{29}C_{27}H_{46}O$. This substance has a solubility of 14 mg in 100 ml 95% ethanol at 18°C. An essential structural feature for the occurrence of this reaction is the presence of the C_3 hydroxyl group in a β-configuration. Alteration of either the group or its configuration prevents the formation of a digitonide. Since most, if not all, naturally occurring free sterols meet these two requirements, it is obvious that the cholesterol digitonide would be contaminated with any other sterols present in the original solution. Schoenheimer[286] developed a method for separating cholesterol or other unsaturated sterols from saturated sterols. It is based on the observation that sterol bromides are not precipitated by digitonin. Thus, by brominating the mixture, cholesterol adds bromine at C_5 and C_6 and digitonin is used to precipitate the saturated sterols. Cholesterol, free from saturated sterols, can then be recovered from the solution by debromination.

The detection of plant sterols in association with cholesterol is of practical interest from the standpoint of detecting adulteration of milkfat with vegetable fat. In one method[13] the sterols are isolated from the glycerides and acetylated. The acetylated sterols are then subjected to repeated crystallization from alcohol or aqueous alcohol, and the melting point determined. That of pure cholesteryl acetate is 114°C while that of phytosteryl acetate (a mixture) varies from 125 to 137°C. The presence of plant sterols will usually be apparent from the melting point of the first crop of crystals, but confirmation can be obtained by further recrystallizations. If phytosteryl acetate is present, the melting point will rise. Chromatographic methods have also been applied successfully to the detection of foreign sterols in butterfat.[73]

When treated with strong acid under dehydrating conditions cholesterol gives a series of color reactions, the details of which depend upon the conditions employed. An example of such a test is the Liebermann-Burchard reaction, which is obtained by dissolving a crystal of cholesterol in 2 ml chloroform and adding 10 drops acetic anhydride and 1 drop concentrated sulfuric acid. A violet-pink coloration which changes to blue is produced. These reactions require nuclear unsaturation in the steroid; they can be obtained with other unsaturated steroids besides cholesterol, and with a number of other polynuclear hydroaromatic compounds which are either unsaturated or become unsaturated under the conditions of the test.

Cholesterol in Milk.—The quantitative determination of cholesterol in milk has usually involved precipitation with digitonin to separate the cholesterol from glycerolipids and other associated materials not precipitated by this reagent. The steroid can be calculated from the weight of the precipitated complex and this complex can be treated further. A number of micro methods are based on the use of reagents which react with the cholesterol to produce color which is measured with a colorimeter or with a spectrophotometer at selected wavelengths.

Both free cholesterol and cholesteryl esters can be determined by analyses made both before and after saponification of the milk lipids. That made before saponification represents the free cholesterol and that made after, the total cholesterol. Chromatographic methods are now available and can be expected to assume increasing importance.

Cholesterol in milkfat has been reported to be both completely esterified and, by Nataf *et al.*[240], completely free. Others have found it to be partially esterified. Patton and McCarthy[257] have reported concentrations of esters corresponding to from 10 to 15% of the total cholesterol; Nieman and Groot[241] found a variation with season—the highest concentration, in May, was about one-third of the total cholesterol. Intrieri and Badolato[142] found the ratio of free to esterified cholesterol to vary with the fat content of the milk as follows: 3.3% fat milk, 2.0; 1.8% fat milk, 2.9; skimmilk, 9.0.

Patton and McCarthy[257] separated the cholesteryl esters chromatographically from other milk lipids and found the following fatty acid composition (the chain length, colon, number of double bonds, dash, and percentage of the total acids are recorded in sequence): 10:0—1.3, 12:0—1.6, 14:0—4.4, 15:0—2.8, 16:0—17.9, 16:1—3.1, 18:0—8.8, 18:1—15.9, 18:2—23.9, 18:3—4.5, 20 to 26—4.5, and 11 unidentified acids—11.3.

Some data regarding the quantitative occurrence of cholesterol in milkfat are given in Table 4.14.

Although cows fed on a special protein-free diet had one-third the

Table 4.14

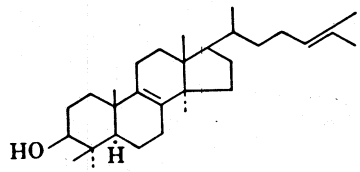
CHOLESTEROL CONTENT OF MILKFAT[a]

Breed of Cow	Fat Content of Milk, %	Total Cholesterol, %
Holstein	2.4	0.413
Holstein	3.3	0.288
Jersey	4.9	0.288
Jersey	5.0	0.300
Jersey	3.9	0.297
Guernsey	3.0	0.346
Guernsey	3.7	0.278

[a] Data calculated from reference 240.

blood cholesterol of cows on normal diet, the total cholesterol in "zero" milkfat was higher—413 mg/100gm compared with 317 mg/100 gm .[351]

Other Steroids.—Lanosterol, previously obtained only from wool fat and yeast, has been isolated by Morice.[232] This compound is synthesized biologically from squalene and is, itself, an intermediate in the biosynthesis of cholesterol. Its skeleton formula is shown below:

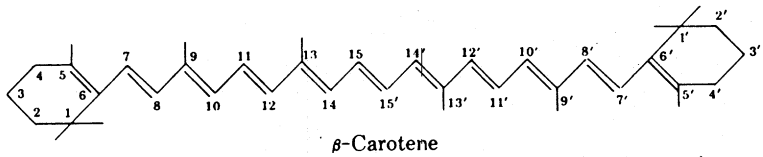


Lanosterol

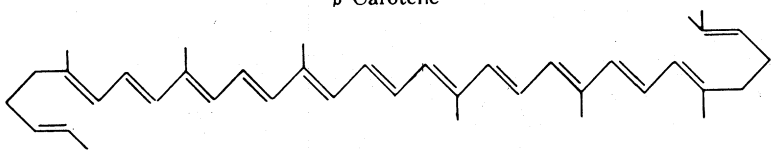
Schwartz *et al.*[292] also found dihydrolanosterol in butterfat. Together, these sterols constituted 0.0046 to 0.0054% of the fat. Brewington *et al.*[36] identified β-sitosterol, a plant sterol, which was present in butterfat at a much lower level than the lanosterols—so low that it would present no complication in the detection of vegetable fat adulterants in butterfat. Parks *et al.*[251] isolated Δ^7 -cholesten-3-one from butterfat and identified it by a variety of means including infrared spectra, mass spectra, and melting point. This work was described as the first report of its occurrence in nature.

Vitamins and Carotenoids.—Milkfat contains vitamins A,D,E, and K and the following carotenoids: α-carotene, β-carotene, γ-carotene, ζ-carotene, lycopene, cryptoxanthin, lutein, zeaxanthine, and neo-β-carotene. The presence of γ-carotene has been inferred but not definitely proved. An extended discussion of the vitamins is given in Chapter 7.

The structures of the various carotenoids can be described on the basis of the structures of β-carotene and lycopene.



β-Carotene



Lycopene

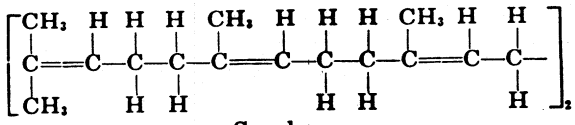
In these structures all the double bonds have the *trans* configuration. The carbon skeleton is considered as being filled out with hydrogen atoms as needed for satisfying the valency requirements of carbon. The lines extended from the skeleton represent attached methyl groups.

β-Carotene is a provitamin A; it is the most active of the carotenoids in this respect. Other provitamins A are α-carotene, γ-carotene, neo-β-carotene, and cryptoxanthin. α-Carotene has the same structure as β-carotene, except that the double bond at the 5′-position is shifted to the 4′-position. γ-Carotene is the same as β-carotene except that one of the rings is open to form a terminal structure as in lycopene. Neo-β-carotene is a mixture of geometrical isomers of β-carotene. The most abundant of these in milkfat appears to be the one with *cis* double bonds at positions 9 and 15. Cryptoxanthin is 3′-hydroxy-β-carotene. All these provitamins A are discussed in Chapter 7.

ζ-Carotene, found in milkfat by Nash and Zscheile[239] is 7,8,7′,8′-tetrahydrolycopene. Lycopene was first reported in milkfat by Gillam and Heilbron.[97] Lutein, found in milkfat by Gillam *et al.*,[98] is 3,3′-dihydroxy-α-carotene. Zeaxanthine, found in milkfat by Frankel *et al.*,[87] is 3,3′-dihydroxy-β-carotene.

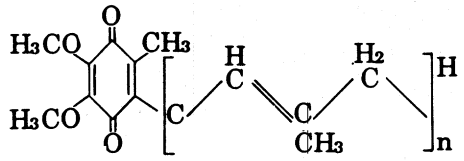
Miscellaneous

Squalene, a liquid acyclic triterpene, was isolated from milkfat by Fitelson[81] in an amount corresponding to 7 mg squalene per 100 gm milkfat. This compound is an intermediate in the biosynthesis of cholesterol. Its formula is as follows:



Squalene

Lopez Lorenzo *et al.*[204] found ubiquinone at a level of 0.817 to 1.17 μg per gm butterfat, and provisionally identified solanesol and dolichol in all samples. Ubiquinone has the following formula:



Ubiquinone (n = 6 – 10)

Solanesol, $H(CH_2\overset{CH_3}{C} = CHCH_2)_9OH$, a constituent of tobacco leaf, has been discussed by Stedman.[325] Dolichol is a long-chain isoprenoid alcohol for which the formula, $H \cdot [CH_2 \cdot C(CH_3){:}CH \cdot CH_2]_{19} \cdot CH_2 CH(CH_3) \cdot CH_2 \cdot CH_2OH$, has been proposed.[41]

Sulfur Compounds.—Although organic esters of sulfuric acid are widely distributed in nature, it is not known whether such compounds are present in milkfat. Trace quantities of organically bound sulfur have been reported.[330] These compounds have not been identified.

The biosynthesis of a number of the compounds discussed in the preceding sections has been reviewed by Goodwin.[103]

FREE FATTY ACIDS

Small proportions of free fatty acids are always present in fresh milkfat; larger percentages are found in the fat isolated from milk or cream that has been subjected to bacterial action or in fat that has been stored for some time. Lipolysis of milkfat, resulting in the production of free fatty acids, is discussed in another chapter. The presence of free acids in unlipolyzed fat may be the result of an incomplete esterification in the mammary gland.

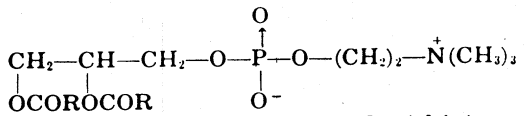
Kintner and Day[177] found the composition of the free fatty acids in fresh milk to be similar to that of the acids bound in the milk glycerides.

Kawashiro *et al.*[166] reported that during storage of butter relatively more of the odd-numbered than of the even-numbered fatty acids are liberated.

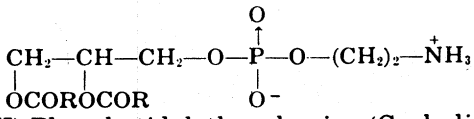
Loney and Bassette[203] studied the liberation of fatty acids during the storage of sterile concentrated milk. Enzymatic lipolysis should not be a factor in this product, so the liberated acids can be considered

a consequence of uncatalyzed hydrolysis of the milk lipids. The rate of fatty acid liberation varied directly with temperature, no detectable lipid hydrolysis taking place during storage at 4°C. The composition of free acids, after storage, was similar to that in the parent lipids.

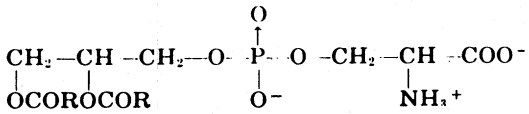
PHOSPHOLIPIDS AND SPHINGOLIPIDS



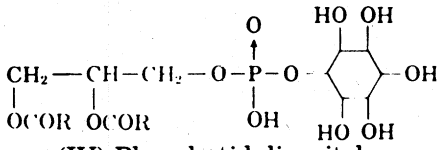
(I) Phosphatidylcholine (Lecithin)



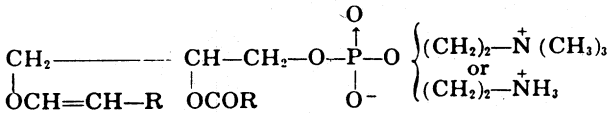
(II) Phosphatidylethanolamine (Cephalin)



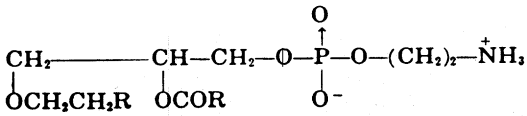
(III) Phosphatidylserine



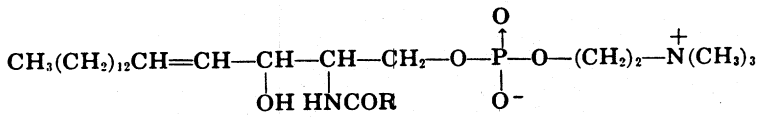
(IV) Phosphatidylinositol



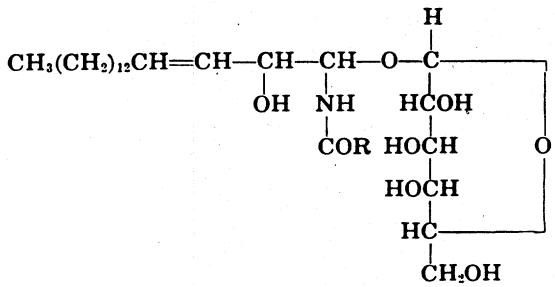
(V) Phosphatidalcholine or ethanolamine (Plasmalogen)



(VI) Phosphatidic glyceryl ether



(VII) Sphingomyelin



(VIII) Cerebroside—a glycosphingolipid

Formulas I-VII are phospholipids and VII-VIII are sphingolipids, sphingomyelin being a member of each group. In addition to variation in their acyl components, 31 long-chain bases in bovine milk sphingomyelin[235] and 33 in bovine milk glycosphingolipids[236] have been identified. The latter group also has variation in its sugar moiety.

General

The phospholipids are widely distributed; they appear to be components of all cells. Proposals regarding their biological functions include the following:[107] implication in the blood-clotting process; as storage forms for fatty acids and phosphate; as a source of choline in nervous tissue; as essential structural elements in living cells; as integral components in biological oxidations; as intermediates in the transport, absorption, and metabolism of fatty acids; and in the transport and utilization of sodium and potassium ions.

For many years the substance referred to as cephalin was thought to be represented by Formula II, above. Folch,[83] however, showed that brain cephalin is a mixture. In addition to phosphatidylethanolamine, he isolated phosphatidylserine and an inositol-containing phospholipid. The term "cephalin" is, accordingly, less specific than nomenclature which indicates the chemical structure.

The above formulas I-VI represent compounds which, if partially hydrolyzed, would produce α-glycerophosphoric acid, in which the phosphoric acid is attached to one of the terminal carbon atoms of

glycerol. α-Glycerophosphoric acid contains the elements essential for optical activity; β-glycerophosphoric acid, in which the attachment is through the middle carbon atom of glycerol, is optically inactive. Although partial hydrolysis of phospholipids has yielded both α- and β-glycerophosphoric acid, this cannot be considered proof that both structures are present in the naturally occurring phospholipids, since hydrolysis of phospholipids is accompanied by migration of the phosphoric acid radical to form an equilibrium mixture of the α- and β-glycerophosphoric acids.[19] Evidence from other sources has been obtained for the occurrence of the α-attachment in the natural phospholipids, but not for the β-attachment.

Almost without exception the natural phospholipids are the isomers in which the phosphoric-acid moiety is attached in the 3-position of *sn*-glycerol. The known exception[157,163] occurs in phospholipid components of the extremely halophylic bacterium, *Halobacterium cutirubrum,* in which the phosphoric-acid moiety is attached in the 1-position of *sn*-glycerol. Another unusual feature of these phospholipids is that they contain no acyl groups but, instead, are diethers.

It is noted that the base, sphingosine, in the sphingomyelin molecule has two hydroxyl groups through which the phosphoric acid radical could conceivably be attached. Evidence for the attachment as shown through the terminal hydroxyl (α-attachment) has been furnished by Rouser *et al.*[277] and by Marinetti *et al.*[214] Carter and Fugino[44] have reviewed the evidence that sphingosine is D*s*-erythro-1,3-dihydroxy-2-amino-4-*trans*-octadecene.

The plasmalogens are lipids characteristic of the animal kingdom which generate aldehydes on acid hydrolysis. The structure of phosphatidic plasmalogens is shown by formula V, above. The linkage to glycerol as an α,β-unsaturated ether was established by hydrogenation[269] and ozonolysis.[55] Hydrogenation formed a saturated ether which no longer had aldehydogenic properties. Ozonolysis produced long-chain aldehydes, a result consistent with a vinyl ether structure.

The position of attachment of the aldehydogenic group has been the subject of considerable controversy. Some evidence is available indicating that both positions of attachment may exist in natural plasmalogens; the predominance of current evidence, however, favors α-attachment. Part of the earlier confusion has resulted from hydrolysis studies employing lecithinase A. This enzyme had been thought to be specific in hydrolyzing fatty acids from the α-position of phospholipids. Tattrie,[337] however, has presented what appears to be unequivocal evidence that the opposite is true, and that lecithinase A, now known as phospholipase A, is specific in hydrolyzing fatty

acids from the β-position. Plasmalogens have been hydrogenated and the resultant saturated ether hydrolyzed to form an acid-free glyceryl ether. Ethers formed in this manner have been shown to be attached through the α-position of glycerol. This has been done by the formation of ethers of known structure,[215] as well as by infrared spectra and periodate-oxidation analysis.[56]

Norton *et al.*[243] have prepared the α-1-alkenyl glyceryl ether from pure phosphatidalcholine. The infrared spectrum indicated that the double bond has a *cis* configuration.

Formula VI, above, represents a saturated counterpart of phosphatidalethanolamine. Such a compound has been isolated[45] from egg yolk. The α-attachment was deomonstrated by periodate-oxidation analysis and proton magnetic resonance studies.

Another type of structural variation, differing from those just discussed, is that involved in acid-base equilibria. In the formulas above, the dipolar ionic forms have been used for those compounds containing both acidic and basic elements. In compounds such as phosphatidylethanolamine, the contribution of the dipolar ionic form is a maximum at the isoelectric point. The presence of excess hydroxyl or hydrogen ions leads to an increased representation of those forms containing a nonionized basic or acidic group, respectively.

However, in choline-containing compounds, such as lecithin, the nitrogen atom is not attached to an ionizable hydrogen atom which could be removed by hydroxyl ions to form a nonionized base. Also, according to the Pauli exclusion principle, this nitrogen atom is not capable of attaching an additional atom or group through a covalent bond. It cannot, therefore, combine with a hydroxyl ion to form a nonionized base. It would thus seem that the choline group must remain ionized, regardless of variations in acidity. Ionization of the phosphate radical is in all instances influenced by the acidity.

Baer and Maurukas[20] obtained analyses of synthetic lecithin which they felt were consistent with the nonionized forms of both the phosphate and choline radicals. The same analyses would be expected, however, if the ionized form contained one molecule of water of crystallization.

Properties.—The phospholipids are not truly soluble in water but can be emulsified to form colloidal solutions or suspensions. Lecithin and cephalin, in addition, are hygroscopic. In general, phospholipids are soluble in fat solvents. There are several exceptions to this statement; for example, they have low solubility in acetone. This property has been widely used in separating phospholipids from fats. Also, sphingomyelin has low solubility in ether, and this fact is utilized in its isolation from other phospholipids. Classically, cephalin has been

isolated by its limited solubility in alcohol. It is now known that pure phosphatidylethanolamine is quite soluble in alcohol, phosphatidylserine is less so, and the inositol phospholipids are the least soluble in this solvent.

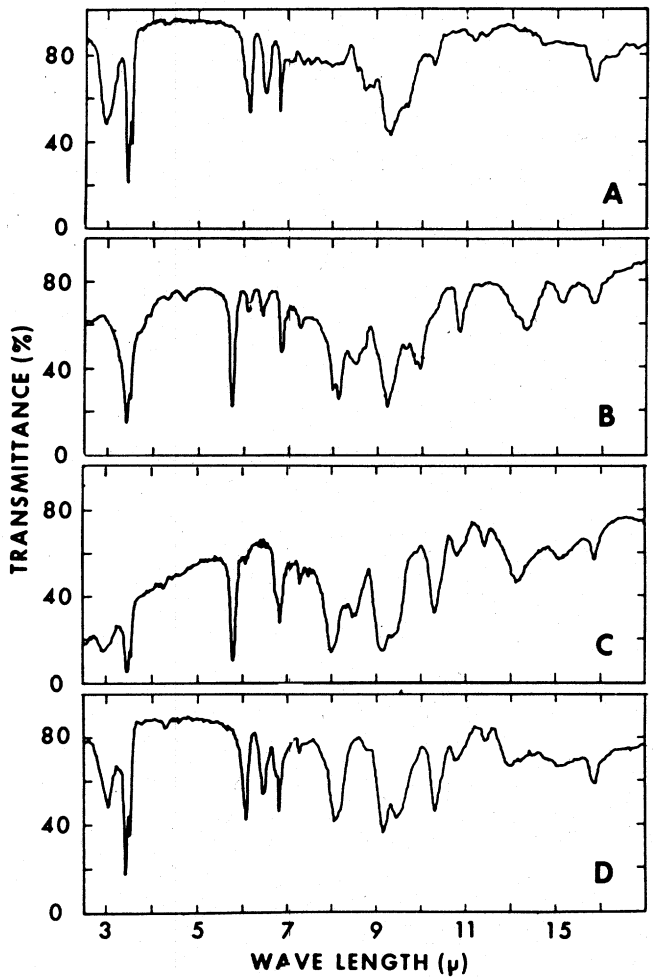
It should be pointed out here that these solubility relationships are influenced considerably by both the nature of the fatty-acid constituents of the molecule and by the presence of impurities. Thus, while lecithin is ordinarily soluble in ether, if its fatty acids are completely saturated it is insoluble in this solvent.[359] The presence of triglycerides increases the solubility of phospholipids in acetone. The presence of inositol phospholipids decreases the solubility of phosphatidylethanolamine in alcohol.

Chemically, the reactions of phospholipids are those to be expected from a consideration of their constituents. It is notable that unsaturated acids combined in phospholipids are more susceptible to oxidation than if combined in a triglyceride.

Preparation and Determination.—The preparation and purification of phospholipids in earlier work have been based largely on the solubility relationships mentioned above, together with the use of complexes with inorganic salts such as cadmium chloride. These relationships are still occasionally employed in preliminary steps, but today chromatographic procedures are in general use. Marinetti[213] has reviewed in some detail the problems involved in isolating phospholipids from natural sources, and in their chromatographic separation, identification, and analysis.

The identification and estimation of phospholipids may be based on a determination of characteristic constituents such as lipid phosphorus, lipid nitrogen, choline, or ethanolamine. Spectral methods are being used increasingly for identifying phospholipids and for determining their purity. These methods are also being applied to their quantitative estimation. Details are given elsewhere.[213,359] Figure 4.5, taken from the work of Smith and Freeman,[316] shows infrared spectra of a cerebroside and of three classes of phospholipids. A correlation between chemical groups and absorption peaks has been illustrated in another publication.[318] Cerebrosides and the phospholipid, sphingomyelin, contain an amide linkage. They can be distinguished from other phospholipids by the strong absorption bands at 6.1 and 6.5 μ that are characteristic of monosubstituted amides. The spectra of both lecithin and cephalin show absorption peaks at 5.8 μ due to an ester carbonyl group, which is lacking in sphingomyelin. Lecithin has a strong absorption peak at 10.3 μ which serves to distinguish it from cephalin.

A suggestion of the complexity of this fraction of the milk lipids



Adapted from Smith and Freeman[316]

FIG. 4.5. INFRARED ABSORPTION SPECTRA OF A CEREBROSIDE AND VARIOUS PHOSPHOLIPIDS

A—Phrenosine. B—Cephalin. C—Lecithin. D—Sphingomyelin.

appeared in early work,[191,192] which indicated the presence of lecithin, cephalin, sphingomyelin, and cerebrosides. Morrison *et al.*,[237] in a summary of later work, reported the following distribution of the major classes of compounds present in this fraction of bovine milk lipids: 30% phosphatidylethanolamine, 28% phosphatidylcholine, 8% phosphatidylserine, 5% phosphatidylinositol, 19% sphingomyelin, 1% phosphatidalethanolamine, 3% phosphatidalcholine, and 3% each of

the sphingolipids, ceramidemonohexoside and ceramidedihexoside. Comparative distribution data are also available[234] for the phospholipids of the cow, ewe, Indian buffalo, camel, ass, sow, and human.

The phospholipids have been reported as generally existing in milk combined by semilabile and labile linkages to protein and carbohydrates.[91] The carbohydrates, which can be retained by proper methods of isolation, are reported to be very easily broken by polar solvents.

Phospholipids in Milk

The data in Table 4.15 are indicative of the levels of phospholipids in milk and some milk products. Approximate levels of individual phospholipid and sphingolipid classes in the total bovine milk lipids can be calculated from the figure 0.87% of this table and the distribution data given above.

Morrison and associates[234,237,238] have studied the effect of species on the fatty-acid composition of milk phospholipids. Data on the acids of the bovine milk glycerophospholipids are given in Table 4.16 and of bovine milk sphingomyelin in Table 4.17. Morrison[234] summarized their findings in the following manner. The ruminant herbivores (camel, cow, sheep, Indian buffalo) have branched-chain acids in all phospholipid classes, they have only a few percent of fatty acids with more than two double bonds, and their sphingomyelin contains about 30% of 23:0 and only a small percentage of 24:1. The nonruminant herbivore (ass) has larger percentages of 18:3, and its sphingomyelin has only a low percentage of 23:0 but about 16% of 24:1. The nonherbivores (pig and human) have less 18:3 than the ass and larger proportions of long-chain polyunsaturated acids. Camel-milk phospholipids are somewhat atypical of their group and contain the exceptionally high level of 15% plasmalogens.

Table 4.15

PHOSPHOLIPID CONTENT (PERCENT) OF MILK AND MILK PRODUCTS[a]

Product	Phospholipids in Product	Fat in Product	Phospholipids in Fat
Whole milk	0.0337	3.88	0.87[b]
Skim milk	0.0169	0.090	17.29
Cream	0.1816	41.13	0.442
Buttermilk	0.1819	1.94	9.378
Butter	0.1872	84.8	0.2207

[a] Data calculated or taken from reference 135.
[b] The reported value of 0.0869 appears to be a misprint.

Table 4.16

COMPONENT ACIDS OF BOVINE MILK GLYCEROPHOSPHOLIPIDS: THEIR LEVELS AND SPECIFIC DISTRIBUTION[a,b]

	Phosphatidylethanolamine		Phosphatidylserine		Phosphatidylcholine	
Acid[c]	1-position	2-position	1-position	2-position	1-position	2-position
10:0					0.2	0.7
11:0	0.1	0.2	0.5	0.1	trace	0.1
12:0	0.3	0.6	1.1	0.5	0.3	0.8
13:0	trace	0.1	0.4	0.1	0.1	trace
14:0i	0.2	0.1	0.1	0.1	0.2	0.1
14:0	1.9	1.3	4.0	2.2	5.6	10.8
15:0i	0.1	0.1	0.3	0.2	0.3	0.4
15:0ai	0.3	0.3	0.5	0.5	0.3	0.6
15:0	0.6	0.4	0.6	0.5	1.6	2.4
15:1	0.1	0.1	0.4	0.3	0.2	0.3
16:0i	trace	0.1	—	0.2	0.4	1.0
16:0	19.7	4.7	24.5	7.6	41.9	30.6
16:1	1.2	2.2	1.7	2.3	0.6	1.2
17:0i	0.4	0.2	0.3	0.2	0.4	0.5
17:0ai	0.4	0.3	1.3	0.5	1.0	1.0
17:0	1.3	0.9	1.3	0.7	1.3	0.6

17:1	0.3	0.6	0.7	0.6	0.2	0.3
18:0i	0.1	trace	0.1	0.1	0.1	0.1
18:0	19.0	1.3	28.0	3.9	17.5	2.4
18:1	45.8	47.8	25.8	46.2	20.3	27.8
18:2	2.9	21.4	1.6	18.3	2.7	9.2
18:2c,tt	0.5	0.2	—	0.4	0.3	0.5
18:3	1.1	4.5	1.2	3.0	0.8	1.8
18:3c,ttt	0.4	1.8	—	1.0	1.1	1.0
19:0	0.4	0.4	0.7	0.4	0.6	0.2
20:0i	0.1	0.1	—	0.2	0.1	0.1
20:0	0.7	0.1	1.0	0.2	0.3	0.2
20:1 ?	0.1	0.2	—	0.1	0.1	0.2
20:3	0.2	2.2	1.2	2.0	—	1.6
20:4	0.2	3.0	1.1	2.7	0.2	1.2
21:0	0.1	0.1	—	0.4	—	—
21:1	—	0.3	1.5	0.6	0.2	0.3
22:0	0.1	0.1	—	0.4	—	—
Unknowns	1.1	3.7	—	4.0	1.1	2.0
Total Saturated	46	11	65	19	72	53
Total Unsaturated	54	89	35	81	28	47

[a] Data from reference 237.
[b] Levels expressed as mole % of total acids in 1- and 2-positions of *sn*-glycerol.
[c] Acid designated by number of carbon atoms, colon, number of unsaturations. Abbreviations: i-iso, ai-anteiso, c-conjugated, t-*trans*.

Table 4.17

COMPONENT ACIDS OF BOVINE MILK SPHINGOLIPIDS[a]

Acid[b]	Percent Composition[c]					
	Sphingomyelin		Glucosylceramide		Lactosylceramide	
	Fatty Acid	2-Hydroxy Acid	Fatty Acid	2-Hydroxy Acid	Fatty Acid	2-Hydroxy Acid
12:0	0.1				0.1	
14:0	0.4	1.6	1.0	2.8	0.3	0.6
15:0	0.1		0.3		0.1	
16:0	7.8	9.2	9.3	12.6	7.7	10.5
16:1		0.8	1.4		0.3	
17:0	0.3	1.1	1.3	1.3	0.2	1.1
18:0	1.6	6.2	13.7	8.4	3.3	3.7
18:1	0.2	0.7	12.2		1.3	
18:2	0.2		2.0		0.2	
19:0	0.2	0.6	1.3		0.2	
20:0	0.6	0.6	0.9	4.5	1.1	1.2
21:0	0.9	2.0	1.2	1.3	1.4	0.9
21:1			0.1			
22:0	20.7	17.2	17.0	16.7	24.9	15.4
22:1	0.7		0.9		0.6	
23:0	30.4	31.5	22.0	31.0	29.5	26.9
23:1	5.0	2.5	3.4	1.7	6.6	6.3
24:0	22.8	21.8	9.9	19.7	16.5	29.5
24:1	4.0	1.5	2.1		3.7	4.1
25:0	1.6	1.9			0.7	
25:1	1.6	1.0			1.4	
26:0	0.8					
Total Unsaturates	11.7	6.5	22.1	1.7	14.1	10.4

[a] From reference 236

[b] Acid designated by number of carbon atoms, colon, number of double bonds.

[c] With reference to each type of acid. The 2-hydroxy were less than 1% of total acids in each lipid class.

The positional distribution of fatty acids in phosphatidylcholine and phosphatidylethanolamine was studied. The acids generally fit a pattern of increasing preference for the α-position with increasing chain length of the saturated acids, and an increasing preference for the β-position with increasing unsaturation. The fatty acids in the β-position of milk phospholipids differed significantly in composition from those in the β-position of the corresponding neutral triglycerides. This was considered support for the idea that the biosynthesis of neutral triglycerides and phospholipids does not proceed by simple direct routes from a common precursor.

Phosphatidic Glyceryl Ethers.—On questionable evidence,[91] these compounds were reported present and were thought to represent the entire glyceryl ether fraction of milkfat. However, glyceryl ethers have been found in butterfat (which is free from phospholipids) at the same level as in the total milk lipids.[291,294]

Phosphatidic Plasmalogens.—Various plasmalogen levels, up to 4% of bovine milk phospholipids, have been reported. Duin[69] found 0.4 to 0.8% bound aldehydes in the phospholipid fraction. This corresponds approximately to 1.3 to 2.5% of the phospholipids. Duin found that the aldehydes were a complicated mixture with chain lengths of 14 to 18 carbon atoms. Besides saturated aldehydes he found unsaturated and probably branched-chain aldehydes. Stearaldehyde and palmitaldehyde were identified with certainty. In addition, there were indications of the following: an α,β-unsaturated C_{18} aldehyde, a C_{18} aldehyde with unsaturation in some other position, a normal or branched C_{17} aldehyde, an α-β-unsaturated or branched C_{15} aldehyde, and a C_{14} aldehyde.

The biosynthesis of phospholipids will not be discussed here except to mention that phosphatidic acid, α',β-diacyl-α-glycerophosphoric acid, is a common intermediate from which either triglycerides or phospholipids may be formed. It is of interest in this connection that there are two series of naturally occurring compounds which differ only in the nature of the substituent on the α-carbon atom of glycerol. These are the neutral series and the phospholipid series. Examples of the neutral series and their phosphatidic analogues are: (1) triglycerides and the phospholipids represented by formulas I, II, III, and IV; (2) neutral plasmalogens and phosphatidic plasmalogens, formula V; and (3) neutral glyceryl ethers and phosphatidic glyceryl ethers, formula VI. As discussed above, however, there is evidence[234] that if the two classes of compounds are derived from a common precursor, their biosynthesis does not proceed by simple direct routes.

For further information the reader is referred to works discussing phospholipids with reference to their structure,[107] their isolation and analysis,[107,213] their biosynthesis,[173,276] and their function.[276]

Sphingolipids in Milk

In addition to a variation in component fatty acids, sphingolipids are characterized by a great variation in their long-chain base moiety. Karlsson[159] has reviewed the sphingolipid long-chain bases with regard to their nomenclature, distribution, metabolism, biologic properties, chemistry, and methods of characterization. His review includes 60 long-chain bases which have been identified or proposed.

Morrison[235] identified 31 long-chain bases in bovine milk sphingomyelin. They consisted of both saturated and unsaturated dihydroxy bases. The saturated bases had normal 12 to 20 carbon atom chains, *iso* 17 to 20 carbon atom chains, and *anteiso* 17 to 19 carbon atom chains. The unsaturated bases had normal 12 to 20 carbon atom chains, *iso* 14 to 19 carbon atom chains, and *anteiso* 15, 17, and 19 carbon atom chains. The unsaturated bases formed 82% of the total.

The long-chain bases in glucosylceramide and lactosylceramide[236] included all those in the sphingomyelin fraction plus two additional: an *iso* saturated and an *iso* monounsaturated 16 carbon atom base.

The fatty-acid and substituted fatty-acid components of bovine milk sphingolipids are shown in Table 4.17. Table 4.18 gives information on the structure of the monoenoic acids of sphingomyelin. Long-chain acids are formed by chain elongation from the carboxyl end of the molecule.[179] The data in this table show a close relationship between the *cis* monoenoic acids of the neutral triglycerides and the longer-chain *cis* monoenoic acids of sphingomyelin. No such similarity is evident between the *trans* monoenoic acids of the two lipids. Table 4.19 shows the high *trans* content of the sphingolipid monoenoic acids.

Obvious differences between the fatty-acid composition of the neutral triglycerides and that of the phospholipid-sphingolipid fraction of milkfat are the absence of short-chain acids in the latter group and the abundance of chain lengths greater than 18 in the sphingolipids. There are also a number of acids present in the sphingolipids which either are absent from the neutral triglycerides or have not been as completely characterized. Notable in this respect are the 2-hydroxy acids listed in Table 4.17 and the numerous positional and *cis, trans* isomers in the 23:1, 24:1, and 25:1 acids of sphingomyelin. Additionally, a conjugated *trans, trans, trans-* 18:3 acid[237] was tentatively identified, and a 22:6 acid[120] was reported present in the phospholipids.

PHYSICAL PROPERTIES OF MILKFAT

Structure

The size of the milkfat globules has been reported to vary from 0.1 to 22 μ in diameter.[59] The majority, however, are within the size range of 1 to 5 μ.

Table 4.18

COMPARISON OF DISTRIBUTION OF DOUBLE BONDS IN CERTAIN *cis* AND *trans* MONOENOIC FATTY ACIDS OF BOVINE MILK SPHINGOMYELIN AND NEUTRAL TRIGLYCERIDES[a]

Position of Double Bond from Terminal Methyl Group	*cis*-Isomers				*trans*-Isomers		
	Sphingomyelin			Neutral Triglycerides	Sphingomyelin		Neutral Triglycerides
	23:1	24:1	25:1	18:1	23:1	24:1	18:1
12		0.3				0.6	
11	1.2	0.1	0.7		1.2	0.8	1.2
10	2.3	1.0	3.0	1.0	2.1	7.5	2.3
9	79.0	96.5	92.5	96.0	59.6	21.1	3.6
8	5.2	1.0	2.8	0.5	9.3	6.5	3.6
7	8.6	—	1.0	2.5	1.7	24.4	45.3
6	2.3	0.3			8.9	5.7	4.7
5	0.7				2.5	11.3	8.8
4	0.7				12.4	19.6	8.8
3					3.2	3.2	8.4
2							13.0

[a] From reference 236. Acids designated by number of carbon atoms, colon, number of double bonds.

Table 4.19

PERCENT *trans* CONTENT OF MONOENOIC FATTY ACIDS IN BOVINE MILK SPHINGOLIPIDS[a]

Acid[b]	Sphingomyelin	Glucosylceramide	Lactosylceramide
18:1	94	44	57
22:1	98	84	84
23:1	64, 70[c]	49	64
24:1	23, 17[c]	20	19
25:1	7, 5[c]	1	2
Total monoenes	44, 46[c]	47, 43[c]	46, 51[c]

[a] From reference 236
[b] Acid designated by number of carbon atoms, colon, number of double bonds.
[c] Values found by different methods of analysis.

The fat globules are stabilized by a surface film containing, among other components, protein and phospholipids. The composition of this film will not be further discussed here as it has been treated in detail in a later chapter. Sommer[323] has pictured the phospholipids as the primary stabilizing force in preventing further growth of the fat globules. According to his conception, triglyceride molecules secreted by the mammary gland, being insoluble in the aqueous phase, combine with similar molecules previously or concurrently formed and thus build up a fat globule. At the same time phospholipids, present in the fluid or simultaneously secreted, deposit in a strictly random fashion on the growing globules. The phospholipid molecules, having both nonpolar and strongly polar groups, orient on the surface of the fat globules with their polar groups in the aqueous phase. When, as a result of this random deposition, the surface of a fat globule is completely covered with phospholipids, it is sealed against the further deposition of triglyceride molecules and so ceases to grow. The protein or proteins of the surface film are then added by complexing with the polar groups of the phospholipids. Sommer has concluded that the phospholipid content of milk is, therefore, just sufficient to seal off the globules against further growth by forming a monolayer on each globule. He has pointed out that this concept affords an explanation of the relationship between globule size and breed of cow and, at the same time, explains the small globules of late lactation. With a rapid formation of triglycerides in high concentration, the globules would grow more than under less favorable conditions before becoming sealed off with phospholipids.

This would explain the comparatively large globules in Jersey milk. In the later stages of lactation, while the concentration of fat in the milk is high, its formation is slow and the phospholipids are relatively

more abundant. Under these conditions the surface would become covered while the globule was still small. Regardless of the correctness of Sommer's views, it is a fact that the milkfat is capable of existence in a state of much greater subdivision than occurs naturally. By homogenization the number of globules has been increased more than 1000 times.

Plastic Properties

Whereas a viscous material will flow under stress—the rate of flow being proportional to the force applied—a plastic material will react to sufficiently small forces by an elastic deformation. Only upon the application of forces greater than a critical value, known as the yield point or yield value, will the material flow. The relationship between the rate of flow and the pressure, while nonlinear, approaches linearity with increased pressure. If a straight line is fitted to this curve in the proper manner it can be used to define the plastic properties of a material such as milkfat.[321] The straight line must fulfill the dual requirements, as closely as possible, of intercepting the pressure axis at 4/3 the yield value and of being asymptotic to the curve in the region of 5/3 the yield value. The slope of such a line, expressed in centipoises, has been referred to by Søltoft[321] as the viscosity, and the intercept, expressed in dynes/cm^2, as the firmness. He used the term "consistency" to express the total plastic properties of a fat.

Various types of instruments have been used to measure the plastic properties of fats. With one type, in which a fat is forced through a capillary tube, measurements such as those pertaining to firmness and viscosity can be expressed in fundamental scientific units. With another type the data can be expressed only in arbitrary units; examples of this type are penetrometers, such as the falling ball or falling needle, and plunger or cutting-wire plastometers.

The response of a plastic material to stresses less than the yield value is at least partially elastic in nature. Thereafter the flow becomes viscous. Since elasticity and viscosity have different dimensions, $ML^{-1}T^{-2}$ and $ML^{-1}T^{-1}$, respectively, they cannot be compared quantitatively nor expressed in a single scientific unit. Søltoft, however, has shown that since a certain relationship usually does exist between firmness and viscosity, it is possible to calculate one of them approximately from the other, and to express the consistency in a single arbitrary unit. This is of practical interest in connection with the use of the instruments mentioned above.

It is also of considerable interest that Scott Blair and co-workers[300] have found that there is no psychological barrier to making comparisons

between viscosity and firmness. In experiments in which rubber balls (showing only elastic behavior) and balls of bitumen (showing only viscous behavior) were manually squeezed for a definite time, the subjects were able to reproduce their descriptions of the relative firmness of the two materials. The materials were rated as equally firm neither when the total deformations at the end of the squeezing time nor when the rates of change of deformation were equal, but rather when some intermediate entity was the same.

Bailey[21] has reprinted photomicrographs of plastic fats showing them to consist of crystals of fat enmeshing a considerable proportion of liquid oil. He has stated that in the range in which they are form-retaining, yet easily workable, fats appear to contain usually about 10 to 30% of solid material. It can be seen in Table 4.20 that milkfat meets these requirements for solids content within the temperature range of about 15 to 25°C.

The plasticity of milkfat and its variation under different conditions are a matter of interest in several ways. Of prime importance is their effect upon the spreadability and palatability of butter and upon its use as a shortening agent in baking.

Of the various factors which affect the plasticity of a fat, perhaps the most important is the proportion of solids present. Søltoft[321] has reported that, on varying the temperature, both the firmness and the viscosity of fats increase logarithmically with an increase in solids content. The solids contents of the fats were measured dilatometrically. This method depends upon the fact that, upon changing the temperature of a material, the volume change associated with a change of phase is greater than that of either the liquid or the solid. So, by heating the material in an apparatus designed to give either a measure of the volume changes at constant pressure or the pressure changes at constant volume, data can be obtained from which it is possible to calculate the proportion of liquid and solid materials. Bailey[21] has discussed the techniques of dilatometry, and has pointed out that this method involves uncertainties which limit its accuracy when applied to unknown mixtures. From dilatometric data, obtained with a Danish butterfat and an American butterfat, he has calculated the percentages of solids in these butterfats at different temperatures. The data are reproduced in Table 4.20.

The plastic properties of a fat depend not only on the proportion of solids but also on the physical properties of its crystals. These, in turn, are to a certain extent dependent upon the history of the fat as regards its thermal treatment and mechanical handling. Bailey[21] has stated his belief that crystals of high-melting glycerides would impart greater plasticity to a fat than would those of lower-melting

Table 4.20

VARIATION OF BUTTERFAT SOLIDS WITH TEMPERATURE[a]

Temperature, °C	Percent Solids	
	American Butterfat[b]	Danish Butterfat[c]
40	0	0
35	2.0	1.5
30	7.5	5.5
25	13.0	9.5
20	22.0	21.0
15	36.0	34.0
10	43.0	40.0
5	47.0	43.0

[a] Data from Table 63, reference 21.
[b] M.p. 37.7°C.
[c] M.p. 38.0°C.

glycerides. He has also pointed out that fats containing large crystals, produced by slow cooling or by other means, are in general softer than the same fats at the same solids content, but with the solids in the form of smaller crystals.

In addition to the factors mentioned, Søltoft[321] has concluded that the effect of temperature on the plastic properties of a fat is due not only to its effect on the solids content of the fat, but also to its effect on the viscosity of the liquid phase. He has attributed 30% of the temperature change of firmness and 50% of the temperature change of viscosity to a change in the viscosity of the liquid phase. While the effect of the viscosity of the liquid phase upon that of the fat might be expected, his conclusions regarding its effect upon firmness appear to be questionable.

Density

The density of milkfat varies with the fatty-acid composition of the triglycerides, with the nature and amounts of nontriglyceride material present in the fat, with the temperature, and with the physical state of the fat. In the introductory paragraph of this chapter reference was made to the fact that the composition of milkfat is altered by dairy processing. In this discussion a distinction will be made between milkfat as it occurs naturally in milk; as it is obtained in bulk by churning, with or without purification beyond that necessary to remove nonlipid materials; and, because of its commercial importance, as it occurs in the fat layer of a Babcock test.

Sommer[323] has disputed reports in the literature that the density

of the fat in milk is related to the size of the globule. He noted that smaller globules have a higher proportion of surface-adsorbed phospholipids than do larger ones, and considered that this probably accounts for the reported density differences. Determination of the total solids or of the milk-solids-not-fat content of milk by means of a fat determination and a lactometer reading made on the whole milk involves the use of an equation containing, in one of its terms, a value for the density of milkfat. Many equations proposed for this purpose were found to be inaccurate if applied to samples other than those used for obtaining the data upon which their derivation was based. Sharp and Hart[303] concluded that a major source of error lay in the determination of the specific gravity of the milk before the fat had reached its phase equilibrium at the temperature of measurement. At the temperatures 15°C and 60°F, one of which is usually specified for specific gravity measurements, the establishment of phase equilibrium in milkfat is especially slow. It is even slower in the fat globules in whole milk than in bulk fat because, due to the isolation of individual fat globules from one another, fat crystals in one particle cannot act as seeds to induce crystallization in the remainder of the fat.

Bulk fat extracted from the milk by appropriate solvents contains the phospholipids associated with it in the whole milk. That obtained by churning has a lower content of phospholipids. Jenness *et al.*[150] conducted an extensive and careful study of the density of milkfat. They obtained the fat by churning, washed it with water, clarified it by centrifuging, dissolved it in acetone, and filtered it. They obtained an average density value of 0.8892 gm/ml at 60°C and an average coefficient of expansion of 78.34×10^{-5} ml/ml/°C for the temperature range 30 to 60°C. This corresponds to a density change of 0.00070 gm/ml/°C for this same temperature range. They were able to conclude that the density of the purified milkfat is relatively constant and is not affected to any marked extent by breed, season, or feed. The same investigators found that the material in the fat column of Babcock tests had an average density of 0.8918 at 60°C and an average coefficient of expansion of 75.58×10^{-5} ml/ml/°C for the temperature range 30 to 60°C.

The data of Sharp[302] (Fig. 4.6) show the density of milkfat for the temperature range 5 to 80°C. Sharp did not describe his method of preparing the milkfat. It is presumed to be similar to that of Jenness *et al.* A literature survey[354] of milkfat densities determined throughout Europe and the United States showed the values to be generally within 0.002 gm/ml of those of Sharp for that part of the curve represented by liquid fat. Values reported at 15°C or 60°F, however, varied over a range of 0.029 gm/ml. It seems probable that this was because measurements were made before phase equilibrium was established.[303]

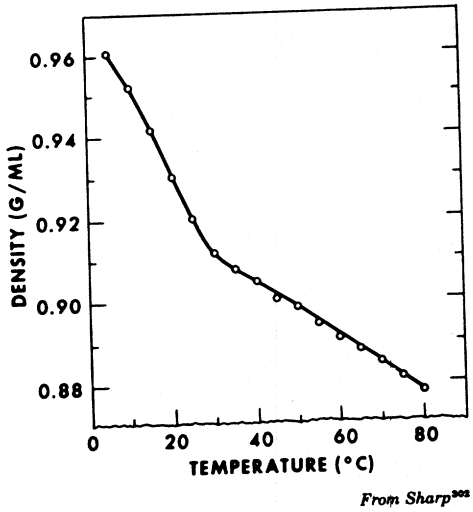


FIG. 4.6. DENSITY OF MILKFAT

METHODS OF EXAMINATION

Milkfat, like other fats, can be characterized by the values obtained from chemical and physical tests. Specific procedures for many of these have been published elsewhere.[229] Some of these tests may be useful in detecting deterioration of the fat; this subject is treated in another chapter. Other tests may indicate the presence of adulterants.

Refractive Index

The refractive index of a fat is influenced by both the molecular weight and the degree of saturation of the component fatty acids. Its determination may be useful as an indication of adulteration but would seldom, in itself, be conclusive.

Iodine Number

The absorption of IBr (Hanus method) and ICl (Wijs method) gives a measure of the unsaturated acids present in a fat. One molecule of halogen compound is absorbed by each unsaturated linkage, and the absorption is expressed as the equivalent number of grams of iodine absorbed by 100 gm fat.

Reichert-Meissl Number

This value is equivalent to the number of milliliters of decinormal sodium hydroxide solution required to neutralize the soluble, volatile

acids obtainable from 5 gm fat. Milkfat contains more of these acids than does any of the fats from which it might be desirable to distinguish it. The Canadian standard for milkfat[43] requires a Reichert-Meissl number of not less than 24.

Polenske Number

This value equals the number of milliliters of decinormal sodium hydroxide solution required to neutralize the insoluble volatile acids obtainable from 5 gm fat. As most of the volatile acids obtainable from milkfat are soluble, this test helps to distinguish between this fat and one of high caprylic acid content (e.g., coconut oil). The Canadian standard for milkfat[43] requires a Polenske number of not more than 3.5 and not greater than 10% of the Reichert-Meissl number.

Saponification Number

The saponification number is equivalent to the number of milligrams of potassium hydroxide required to saponify 1 gm fat. A butterfat that yields a high Reichert-Meissl value also gives a high saponification value, though the latter may drop considerably in the last stages of lactation. The two tests should be collated. A high saponification value and a low Reichert-Meissl value are grounds for suspicion of adulteration.

Some characteristic values of milkfat are given in Table 4.21. It is realized that data not covered by the author, if included, might alter the variation limits shown in this table.

Detection of Adulterants in Milkfat

There are two general ways in which adulteration of milkfat can be established. One involves the identification of a foreign material in the fat. The other involves the demonstration that a normal component of milkfat is present in proportions not encountered in authentic samples. The first method seems to be restricted to the detection of adulteration by vegetable fats.

The difference between the sterols of animal and vegetable fats affords a positive means for identifying adulteration by a vegetable fat. Phytosterol is not a single compound, however, but a mixture. Some of its forms give acetates with chemical and physical properties much closer to those of cholesteryl acetate than do the others, and may not be detectable by the crystallization method with certainty unless the adulterant is present in relatively high proportions. A much more sensitive method for detecting plant sterols has been described

Table 4.21

RANGE OF CHARACTERISTIC MILKFAT VALUES

Refractive index	1.4527–1.4566
Iodine no.	26–28
Saponification no.	220–241
Reichert-Meissl no.	23–33
Polenske no.	1–3

by Eisner *et al.*[73] The sterols of butter and of margarine oils were isolated and fractionated by chromatography of the unsaponifiable fractions on Florisil columns followed by gas-liquid chromatography of the sterol fractions. Cholesterol was the only sterol component found in any butter sample. Stigmasterol, β-sitosterol, and γ-sitosterol were found in all margarine samples. This method was successful in detecting as little as 1% margarine in butter.

Vitamin E exists in several modifications. Brown[39] found only α-tocopherol in cow's milk, while most of the vegetable oils he examined also had β-, γ- or δ-tocopherol. The Canadian standard[43] requires that the tocopherol content shall not exceed 50 μgm/gm of fat.

The detection of animal fat adulterants is less positive in that it depends upon establishing a quantitative change in the composition or the physical constants of the milkfat. The high proportion of the soluble, volatile fatty acids, particularly butyric acid, is the most prominent difference between that of milkfat and other animal fats. Tests such as the Reichert-Meissl number, the Kirschner number, and the direct determination of butyric acid can be used to establish gross adulteration of milkfat. With smaller proportions of adulterants the quantitative differences in these values of course become less pronounced. Legal proof, in such instances, is made still more difficult by the occasional reports in the literature of extreme values for butyric acid content and for such tests as the Reichert-Meissl number.

Of great interest in connection with the detection of adulterants in milkfat are the surveys of United States butterfat constants by Zehren and Jackson[363] and by Keeney.[167] Zehren and Jackson determined the Reichert-Meissl, Polenske, and refractive index values monthly for one year on butterfat obtained from 42 sources from 6 geographical areas having a coverage of 25 states. They analyzed a total of 500 samples. The Reichert-Meissl values ranged from 24.24 to 33.55, with an average value of 28.99. The Polenske values varied from 1.12 to 2.95, with an average value of 1.93. The refractive index varied from 1.4531 to 1.4557, with an average value of 1.4540. Keeney analyzed the samples for butyric acid content. He found that this value

varied from 9.6 to 11.3 mole %, with a mean value of 10.41 mole %.

High-concentrate, low-roughage diets, as pointed out earlier, may decrease the proportion of butyric acid quite markedly. This introduces a further complication into the problem of detecting adulterants, as authentic milkfat from cows on such diets may have uncharacteristically low butyric acid values. Similarly, the high levels of linoleic acid that can now be introduced into milkfat may further distort the characteristic pattern of fatty acid composition. Bhalerao and Kummerow[25] have approached the adulteration problem from the viewpoint that it is more difficult to simulate the glyceride structure of milkfat than its fatty-acid composition. They proposed a method based upon a preliminary fractionation of the glycerides. Kuksis and McCarthy[186] have investigated analysis of the triglycerides by gas liquid chromatography as a means of detecting adulteration.

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