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# Structural Analysis of Natural Triglycerides

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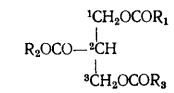
An account is given of modern views on the structural analysis of triglycerides of natural fats by transesterification, lipase hydrolysis, determination of stereoisomerism, and establishment of polymorphism in their crystals. The possibilities of the practical application of these methods in fat analysis are shown. A list of 172 references is included.

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## I. INTRODUCTION

It was assumed for a long time that natural fats were mixtures of simple monacid triglycerides (I) in which the alkyl groups R were identical ($R_{(1)} = R_{(2)} = R_{(3)}$).



(I)

At the beginning of the twentieth century, however, mixed-acid triglycerides based on (I: $R_{(1)} \neq R_{(2)} \neq R_{(3)}$, $R_{(1)} \neq R_{(3)}$) were isolated from several fats. The problem of the structure of triglycerides arose. The hypothesis of a statistical distribution of acyl residues RCO in triglycerides, according to which the probability of formation of a particular individual triglyceride was determined solely by the molar concentration of its component fatty acids in the given fat and by the frequency of occurrence of these acids in the given triglyceride was soon abandoned, and the search for the laws governing the glyceride structure of natural fats has continued to the present time.

The present Review makes a systematic survey of the development of work on the positional distribution of acyl groups in triglycerides—from the original discovery by transesterification of the non-statistical character of the structure of natural fats to the establishment by lipase hydrolysis of the non-equivalence of the 1,3- and the 2-positions in the glycerides, and finally to the recent discovery of the stereospecificity of the structure of animal and plant triglycerides. The last section of the Review deals with the polymorphism of glycerides, which, in combination with their chemical synthesis (Section II, 3), is also used to establish the structure of individual triglycerides in natural fats.

## II. ESTABLISHMENT BY TRANSESTERIFICATION OF THE NON-STATISTICAL DISTRIBUTION OF ACYL RESIDUES IN NATURAL TRIGLYCERIDES

### 1. Transesterification of Triglycerides

Transesterification is the free redistribution (randomisation) of acyl groups among glycerides under the influence of certain physical and chemical factors, as a consequence of which the structure of the glycerides becomes statistical instead of oriented. It is the oldest method in the structural chemistry of natural triglycerides, for the discovery of marked changes in melting point and other properties of fats after randomisation was the immediate reason for posing the problem of their structure. During actual transesterification the initial and final products are triglycerides: depending on whether the acyls migrate within a single glyceride or between different triglycerides, we can distinguish intramolecular and intermolecular transesterification. Glycerolysis—the transesterification of a fat with excess of glycerol—results in the appearance of a large quantity of mono- and di-glycerides. Transesterification is of great practical as well as theoretical value: the changes in polymorphism, melting point, texture, plastic range, and other properties of a fat accompanying randomisation and glycerolysis greatly influence its digestibility and its suitability for the manufacture of margarines, monoglyceride emulsifiers, and other technical products having specified properties [1-6].

Transesterification can be accomplished [6-31] by treating a fat free from moisture, free acids, and peroxides in several ways—(1) in the absence of a catalyst at 300°C, (2) with alkaline catalysts at 100–140°C with 0.03–0.3% of sodium methoxide, at 30°C with 0.6% of sodium methoxide for 1 h (for a 3:2 mixture of an oil and hexane), or at 20°C with 0.005% of lithium t-butoxide for 12–15 h (for a 1:10 mixture of a fat and dimethylformamide), (3) with naphthalene-2-sulphonic acid as catalyst, or (4) with tin and its salts or dihydroxide at 225°C [2,3,6,9,10]—after which the catalyst is neutralised, and the glycerides are purified [11]. The most effective catalyst is sodium methoxide, but at high temperatures complete randomisation of the composition cannot be obtained, though under milder conditions a purely statistical mixture of glycerides is produced [13]. A statistical mixture of triglycerides can also be obtained by transesterification of the methyl esters of fatty acids with triacetin ($Ac_3$), [8] but the transesterification of cocoa-butter with $^{14}C$-tristearin and tritium palmitate yields uniformly labelled $^{14}C$- and $^{3}H$-triglycerides [12]. Lagrange's rule of multipliers for a system of any two fatty acids A and B shows that the maximum content $[A_2B]$ of a glyceride $A_2B$ is reached when $[A] = 2[B]$; we then have the contents 44.4%, 29.7%, 22.2%, and 3.7% for $[A_2B]$, $[A_3]$, $[AB_2]$, and $[B_3]$ respectively. These calculations are used in selecting the acid concentrations for the preparation of a mixture of glycerides of a specified composition [4,7].

As already mentioned, the properties, composition, and structure of triglycerides are greatly changed by transesterification. The changes in the Bömer number and in the melting point depend on departures from the random values in the concentrations in a given fat of glycerides containing only saturated acyls [$Sat_3$].[2] Appreciable changes occur also in the phase structure of the triglycerides (Section V, 3). When molten pork fat, containing 25% of 2-palmito-oleostearin (2-PalOlSt), is chilled rapidly, coarse crystals of the $\beta'$-3-polymorphous form of this glyceride are produced, and prevent the use of this fat in the confectionary industry. After transesterification the 2-PalOlSt content falls to the statistical value (3.4%), and readily fusible $Sat_2Unsat$ glycerides of vegetable type appear; these crystallise in the $\beta'$-2-form, the granularity of the product disappears, and its emulsifying power improves [4,14]. It is possible also to change the properties of a fat in the required direction by controlled transesterification, in which the randomisation of liquid fats such as cottonseed oil is combined with low-temperature crystallisation, which removes saturated acids from the mixture as a $Sat_3$ precipitate [4,7].

The transesterification of natural and artificial fats usually increases the total number of individual triglycerides of differing polarity [15-18]. Olive oil yields the new glycerides OlPalLinoleoyl and OlStLinoleoyl, and a mixture of $St_3$ and $Ol_3$ is converted into the more complicated statistical mixture of StStSt, OlStSt, StOlSt, StOlOl, OlStOl, and OlOlOl [11,19]. The structure of transesterified fats is usually close to the statistical structure, independently of the presence of a solvent in the reaction mixture.

However, selective physicochemical reaction parameters (preferential re-esterification of acids having a longer chain, differences in reactivity between 1- and 2-hydroxy-groups of glycerol, etc.) often divert the distribution of acyls in the randomised oil from the calculated distribution [2,19,21]. Such deviations have been found, in particular, in the composition of transesterified fats rich in volatile acids, such as coconut oil and especially randomised milk fat, which differs appreciably from the original fat (Fig. 1A) in its content of triglycerides in which the total number of carbon atoms in all the acyls is $m = 50-54$) (Fig. 1B) [13,19]. The equilibrium mixtures obtained by transesterifying $St_3$, $Ol_3$, and $Ela_3$ (where Ela = elaidate) or a mixture of the methyl esters of the fatty acids with $Ac_3$, have the statistical composition [8,17,22]. The content of fractions of different polarity does not vary so greatly in the transesterification of liquid oils (safflower and olive) as in the randomisation of solid cocoa butter [12, 18,21]. The transesterification of sunflower and cottonseed oils produced statistical distributions of unsaturated acyls in individual groups of triglycerides [23]. The composition of milk fat, pork fat, soya-bean oil, and olive oil after transesterification corresponded to the statistical distribution [10,15]. If the content of saturated acyls at position 2 of the glycerides [$Sat_{(2)}$] is lower after randomisation of a vegetable oil, contamination of the oil with fats obtained by esterification is indicated [20].

## 2. Glycerolysis of Triglycerides

A fat is subjected to glycerolysis by heating it with excess of glycerol in 0.1% caustic soda in a stream of an inert gas. Cessation of reaction and the establishment

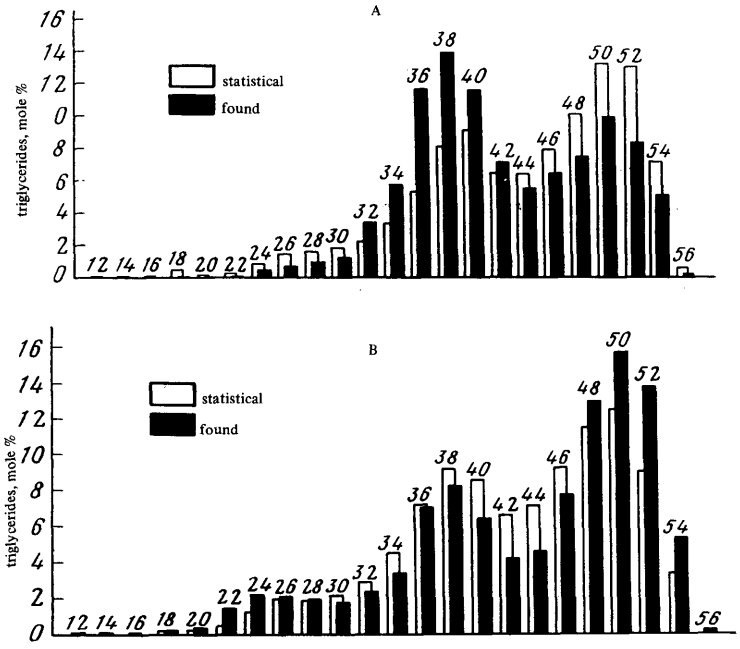



Figure 1. Composition of triglycerides of milk fat [13]: A) native; B) transesterified. The numerals 12-56 indicate the numbers of carbon atoms in individual fractions.

of dynamic equilibrium are assessed from the solubility of the mixture in 2–5 parts of methanol. The equilibrium constant $K_e$ of glycerolysis is calculated from the contents in the mixture of glycerol, 1(3)- and 2-monoglycerides, 1,3- and 1,2(2,3)-diglycerides, and triglycerides determined by adsorption chromatography, measurement of the hydroxyl, acid, and saponification numbers, and also periodate oxidation [16,24]. Such calculation established a fundamental fact in triglyceride chemistry: positions 1 and 2 are not equivalent with respect to acylation [25–29].

Table 1. Composition (wt. %) of equilibrium mixture of glycerolysis* products [24].

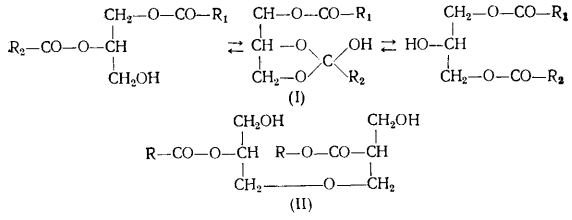
| Components | Found | Calculated for $K_e$=3.4 | Calculated for $K_e$=1 |
|---|---|---|---|
| Glycerol | 6.5 | 6.5 | 6.5 |
| 1(3)-Monoglycerides | 36.5 | 39.5 | 28.3 |
| 2-Monoglycerides | 4.4 | 5.1 | 14.2 |
| 1,2(2,3)-Diglycerides | 15.6 | 14.1 | 27.0 |
| 1,3-Diglycerides | 32.7 | 26.9 | 13.5 |
| Triglycerides | 4.3 | 7.9 | 10.5 |

*Linseed oil (1 mole) and glycerol (1.85 mole) were heated with 0.1% trisodium orthophosphate at 240°C for 2 h.

If glycerolysis occurred strictly quantitatively, and the probability of esterification was the same for glycerol derivatives containing free primary and secondary hydroxy-groups ($R^POH$ and $R^SOH$), the molar contents of esterified ($a$) and free ($b$) hydroxyl in the mixture could have been used to calculate the statistical concentrations of tri-, di-, and mono-glycerides and of free glycerol as ($\times 10^{-4}$) respectively $a^3$, $3a^2b$, $3ab^2$, and $b^3$. [4,26] In this case the relative molar concentrations of positional isomers in the monoglycerides and diglycerides at the equilibrium $R^POH + R^SOOCR \rightleftharpoons R^SOH + R^POOCR$ would have been [1(3)-MGl] = 2[2-MGl] and [1,2(2,3)-DGl] = 2[1,3-DGl], while the equations $K_e$ = [1(3)-MGl]/2[2-MGl] = [1,2(2,3)-DGl]/2[1,3-DGl] would give a calculated value of $K_e$ = 1. However, the equilibrium concentrations obtained by the usual glycerolysis (at 175–180°C) were [1(3)-MGl]/[2-MGl] = 88 : 12 and [1,2(2,3)-DGl]/[1,3-DGl] = 40–60, from which $K_e$ = 3.0 ± 0.6. Hence the free energy of a primary hydroxy-group of glycerol on esterification $\Delta F = -RT \ln K_e$ exceeds the free energy of the secondary group by 1.2 kcal mole$^{-1}$, and the probability of esterification of a primary hydroxyl is treble that of the secondary group [26,27]. The composition of the equilibrium mixture calculated with $K_e$ = 3.4 agrees well with the experimental results, but those calculated with $K_e$ = 1 show marked discrepancies (Table 1) [24]. Increase in the number of carbon atoms $m$ and decrease in the number of double bonds $e$ in the acyl residue diminishes the rate of acyl migration, but does not affect the value of $K_e$. [25] Substitution of one primary hydroxyl does not affect the rate of esterification of the other primary group. With fall in the temperature of the reaction $K_e$ increases [28].

Little work has yet been done on the mechanism of transesterification and glycerolysis [30–32]. Transesterification itself may be a particular case of glycerolysis, in which, under the influence of the catalyst and of the free hydroxyls of water as initiators of the reaction, traces of glycerol, monoglycerides, and diglycerides continually appear in and disappear from the mixture, with a consequent dynamic equilibrium (randomisation) of the acid radicals [4]. A cyclic orthoester (I, Scheme 1) may be an intermediate product in the acyl migration [31].

Scheme 1



Study of the migration of the acyl groups in mono- and di-glycerides in ethereal hydrogen-chloride solution has shown that this reaction requires the presence of traces of water. The mixture formed by the transesterification of a 2-monoglyceride contains a 1,7-dihydroxy-4-oxa-heptane-2,6-diyl dicarboxylate (II) (a dimerised ether), and corresponding derivatives are formed by the transesterification of 1(3)-monoglycerides and 1,3-diglycerides. According to one view an equilibrium mixture of the 2-monoglyceride and (II) is produced in the solution, after which rupture of the ether bond occurs with simultaneous intramolecular migration of the acyl group and formation of a 1(3)-monoglyceride [30]. According to another author (II) is not an intermediate but a secondary product of the reaction, which is synthesised by the combination of a monoglyceride or a diglyceride with the corresponding acid chlorides formed by the action of hydrogen chloride on these glycerides [32].

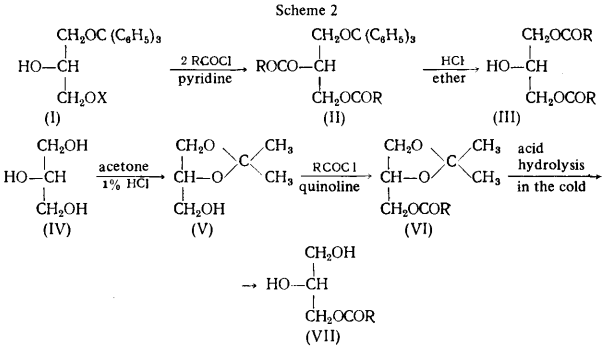
### 3. Chemical Preparation of Triglycerides of Definite Structure

Together with transesterification, the preparation of specified triglycerides (of a mixture of stereoisomers—see Section IV, 8) for comparison with natural triglycerides (melting point, polymorphism, etc.), in order to identify the latter, was one of the earliest methods of investigating fats [33–37]. Monacid triglycerides (AAA) are prepared by the single-stage direct esterification of excess of the fatty acid with glycerol in the presence of toluene-$p$-sulphonic acid. The preparation of diacid and triacid triglycerides is a multistage process with protection of particular hydroxy-groups at individual stages, and also the isolation, purification, and identification of intermediate products [5,34].

Symmetrical triglycerides ABA are obtained by combining 1,3-diglycerides of acid A, which are easily segregated from the more easily fusible 1,2(2,3)-diglycerides by crystallising the equilibrium mixture, with excess of the chloride of acid B by heating in chloroform–pyridine solution [36]. Migration of acyl groups, which is very considerable when toluene-$p$-sulphonic acid is used as catalyst, is absent from this reaction. A statistical mixture of the 1,3-diglycerides of palmitic and stearic acids on reaction with oleoyl chloride gave a mixture of triglycerides similar to cocoa butter [12].

Scheme 2 shows the reactions involved in the preparation of 1,3-diglycerides of polyunsaturated acids (I–III) and 1(3)-monoglycerides (IV–VII). 1,3-Diglycerides of polyunsaturated acids (III) which crystallise with difficulty are prepared by acylating the 1(3)-monotrityl ether of glycerol (I: X = H) and then detritylating with quantitative migration of the 2-acyl to the outer position. Unsymmetrical diacid triglycerides AAB are obtained by using the chloride of acid A to acylate 1(3)-monoglycerides of acid B (VII: R = B), prepared by Fischer's method with 1,2(2,3)-isopropylidene protection subsequently removed by acid hydrolysis [29,38].

Scheme 2




Finally, the preparation of a triacid triglyceride (ABC) involves first obtaining the 1(3)-monoglyceride of acid A (VII: R = A) by the mechanism (IV) → (VII), and then converting it into the 3(1)-monotrityl ether (I: X = COA), which is esterified in the 2-position by the chloride of acid C with subsequent acyl migration by the mechanism (I) → (III) to give the 1,3-diglyceride of acids A and C (III: R = A or C); this is substituted at position 2 by the chloride of acid B. If C is an unsaturated acid, the trityl protection in (II) is removed by hydrogenolysis without acyl migration, and the resulting 1,2(2,3)-diglyceride is acylated at the last stage by the chloride of another unsaturated acid to form a triacid triglyceride of type $SatUnsat_2$. [4,35]

## III. STRUCTURAL ANALYSIS OF TRIGLYCERIDES BY LIPASE HYDROLYSIS

### 1. Lipase Hydrolysis of Triglycerides

It was discovered as early as 1945 that the lipase hydrolysis of fats in the intestine ceased long before all the ester bonds had been cleaved [1,35,39–45]. Subsequent experiments with synthetic triglycerides of known structure showed that 1,2(2,3)-isomers predominated among the diglycerides present in the products of incomplete hydrolysis [39,44]. The preferential detachment by pancreas lipase *in vivo* and *in vitro* of the outer acyls in triglycerides of saturated and unsaturated acids having $m$ = 12–18 is indicated also by the incorporation of $^{14}C$-labelled free fatty acids, in the presence of lipase and a fat, only in the 1,3-positions of triglycerides, and by the formation of the 2-isomers of monoglycerides on lipase hydrolysis, as demonstrated by their isomerisation with perchloric acid and periodate oxidation [40 46–51]. On the basis of these discoveries a method was proposed in 1956 for determining the composition of acyl groups in the 1,3- and 2-positions of triglycerides in natural fats from the composition of the free fatty acids and of 2-monoglycerides resulting from its enzymic cleavage [48]. Lipase hydrolysis is now the principal method for the structural analysis of triglycerides [42,47].

In contrast to esterases, which hydrolyse water-soluble substrates, lipase (glycerol ester hydrolase, EC 3.1.1.3) is almost completely specific to 1,3-esters of glycerol which have limited solubility in water [52,55]. In oil of ergot, for example, it does not cleave the ester bonds between the ricinoleate residues and the glycerol [56]. Furthermore, lipase possesses no stereoselectivity (Section IV, 1). [39] The simplified lipase reaction can be divided into two stages:

$$\text{TGI} \xrightarrow{k_1} \text{1,2(2,3)-DGI} \xrightarrow{k_2} \text{2-MGI} \quad .$$

Each stage has its own rate constant: for the oil from *Madhuca longifolia* the constants are $k_1 = 0.35$ and $k_2 = 0.36$; but the corresponding values for pork fat are 0.31 and 0.36. From the equation for the reaction we find that (where e is the base of natural logarithms and $t$ the time of reaction) [52]

$$[\text{TGI}]_t = [\text{TGI}]_0 \cdot e^{-k_1 t};$$

$$[\text{DGI}]_t = [\text{DGI}]_0 + [\text{TGI}]_0 \cdot [k_1/(k_2 - k_1)]\,(e^{-k_1 t} - e^{-k_2 t});$$

$$[\text{MGI}]_t = [\text{MGI}]_0 + [\text{TGI}]_0\{1 - [1/(k_2 - k_1)]\} \cdot (k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \quad .$$

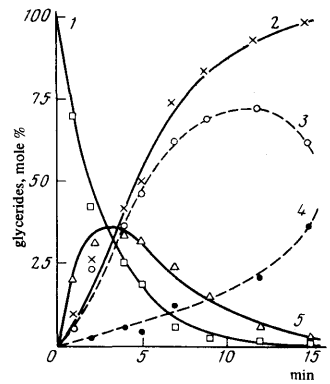



**Figure 2.** Dependence of lipase hydrolysis of oil of the plant Madhuca longifolia on time of reaction [52]: 1) triglycerides; 2) monoglycerides + glycerol; 3) monoglycerides; 4) glycerol; 5) diglycerides. The full curves represent calculated concentrations (Section III, 1), and the broken curves the experimental values.

Before hydrolysis the protein of the enzyme is absorbed on the surface of the fat micelles, and therefore the reaction velocity depends on the degree of emulsification of the substrate in the aqueous solution, which determines the area of this surface, and also on the relative quantities of enzyme and fat [57]. The free fatty acids and monoglycerides formed by hydrolysis are desorbed into the aqueous phase [55]. The rate of the reaction gradually decreases, since triglycerides, 1,2(2,3)-diglycerides, and

2-monoglycerides release 2, 1, and 0 mole respectively of acids per mole of glycerol [53]. Maximum contents of diglycerides and monoglycerides are observed after 40% and 70% hydrolysis respectively (Fig. 2). Free glycerol may appear in the medium after 25–40% hydrolysis, as a consequence of acyl migration (Section III, 3) [47,52]. Several reviews have been published on the properties, kinetics, and mechanism of the reaction of pancreatic lipase [1,41,43,44,54].

Table 2. Reaction conditions (per 1 g of fat) in studying the structure of triglycerides by lipase hydrolysis (data in parentheses relate to the micromodification of the method) [1,10,35,47,55,56,58–64].

| Parameter of reaction medium and units of measurement | Numerical value |
|---|---|
| Composition and concentration of buffer solution | 1.2 M or 1 M $NH_4OH$-$NH_4Cl$ |
| Quantity of buffer solution | 18–23 (160–1050) |
| Optimum pH of buffer solution | 8.0–8.5 |
| Sodium taurocholate, mg | 5–20 (250–500) |
| Calcium chloride, mg | 350–440 (2000–8000) |
| Enzyme, mg | 70–400 |
| Temperature of medium, °C | 37–40 |
| Duration of incubation, min | 10–90 (2–30) |
| Degree of hydrolysis of ester bonds, % | 50–70 |
| pH of medium after addition of 1–6 *N* HCl | 1–3 |
| Atmosphere | nitrogen or air |
| Rate of stirring of mixture, rev/min | 120–3000 |

## 2. Conditions of Lipase Hydrolysis

The conditions under which lipase hydrolysis is conducted are summarised in Table 2. The source of the enzyme is usually the secretion or a preparation of pig pancreas—pancreatin, steapsin, etc.—which may vary greatly in activity and in positional specificity [15,42,56,59]. The quantity of lipase is chosen to give a 65–70% yield on hydrolysis for the minimum time, and its specificity is checked by its action on synthetic triglycerides of known structure [44,47,49,58,64]. The activity of the enzyme increases by a factor of 135 on purification, which includes removal of fat, extraction with water, adsorption on aluminium hydroxide, precipitation with ammonium sulphate, dialysis, and chromatography on DEAE cellulose. However, purification does not improve positional specificity, and for the study of glycerides, therefore, it is usually restricted to Desnuelle's stage (II). [40,43,65,66] It is important to remove endogenous lipids from the preparation, and to eliminate any esterase activity (if glycerides of acids having $m < 12$ are under investigation) and non-specific "lipase" activity, which sometimes amounts to 2–10% of the total lipase activity, is promoted by a taurocholate, and is inhibited after 2 h at pH 9 and 40°C. [62,63,67,68]

The substrate—natural or synthetic triglycerides—and other reactants are also freed from any contamination with mono- and di-glycerides [44,69]. Unsaturated glycerides are hydrolysed in an atmosphere of nitrogen (Table 2). Only a liquid and well emulsified substrate undergoes cleavage [15–39], and therefore the mixture with the emulsifier is homogenised by heating before addition of the lipase. As acyl migration and inactivation of the enzyme increase above 40°C, homogeneity of the medium is best created by rapid mechanical stirring [40,43,47]. The usual emulsifier is sodium taurocholate, which, however, activates the foreign "lipase" (see above) and exerts a still not altogether clear promoting effect on the pancreatic lipase itself [41,59]; polymers—polyvinyl alcohol, gum arabic, etc.—are also used [42,61]. However, the emulsifiers are often inadequate for the production of good emulsions from solid $Sat_3$ glycerides (chain length of each acyl $m_S > 12$); in that case they are mixed, before emulsification, with liquid carrier lipids, which do not influence the specificity of hydrolysis of saturated substrates [70,71]. Mixing an $Sat_3$ glyceride with triolein (1 : 1 or 4 : 1) contaminated the reaction mixture with hydrolysis products of the latter, and therefore it is now replaced by methyl esters—methyl oleate, methyl pentadecanoate, etc. [55,63,69]

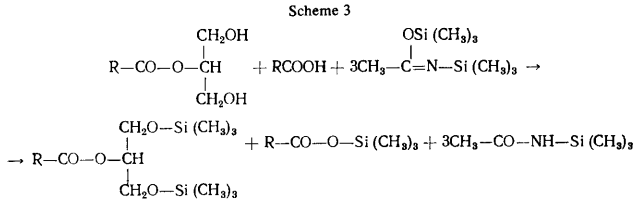
With hydrolysis on a macro-scale, when the molar ratio of fat (0.2–2 g) to buffer is ~ 1.5, the pH falls rapidly below the optimum value (Table 2). [10,47,59,61,63] To avoid inactivation of the lipase the optimum pH must be maintained by adding ammonium hydroxide or 0.5–1 *N* caustic soda either manually or by means of a recording pH-stat, which enables the point of maximum content of monoglycerides in the medium to be detected (Fig. 2). [42,56,59,63,72] In the micro-method (1–50 mg of fat) the existing buffer solution usually ensures maintenance of the optimum pH [47,65]. Calcium ions (Table 2) bind free fatty acids, and therefore hydrolysis proceeds to the monoglycerides; in the absence of the calcium ion, or in the presence of a phosphate buffer to combine with it, only diglycerides are produced, and free fatty acids accumulate and inactivate the enzyme [43,44,55]. If time $t$ elapses between addition of the lipase and the appearance of turbidity due to insoluble calcium salts, time $2t$ corresponds to the maximum content of monoglycerides [41,54]. Reaction is terminated by excess of hydrochloric acid, and the total lipids are extracted [40,57,61,73].

The structure of natural triglycerides is determined from the composition of the products of lipase hydrolysis. The composition of the mono- and di-glycerides can be established by gas chromatography of their acetates: monoglycerides could be separated as far as monoarachin, and diglycerides as far as palmitomyristin. Acetates of 1-monoglycerides and of 1,3-diglycerides were eluted more slowly than the derivatives of 2-monoglycerides and 1,2-diglycerides respectively [74]. A mixture of 1- and 2-monoglycerides was analysed by treatment with methanesulphonyl chloride and an acetone solution of sodium iodide: 2-monoglycerides pass into the 1-isomers, and the resulting allyl esters of fatty acids are separated on the non-polar liquid phase at 240°C for 60 min [75,76]. Quantitative determination of the hydrolysis products of glycerides containing acid-labile groups in the chain of the fatty-acid residues was accomplished by gas chromatography of the unchanged triglycerides and the trimethylsilyl derivatives of monoglycerides, diglycerides, and free aliphatic acids, which were obtained in a 20% solution of bistrimethylsilylacetamide in pyridine (Scheme 3). The trimethylsilyl ethers of monoglycerides and diglycerides and the trimethylsilyl esters of fatty acids were immediately separated by temperature programming. This method was used for the positional analysis of glycerides of *cis*-12,13-epoxyoleic, 10-hydroxyoctadeca-*trans*-9,*trans*-12-dienoic, and octadeca-*cis*-9,*trans*-11,*trans*-13-trienoic acids; decomposition products obtained by other methods of silylation were absent here [77].

The products of lipase hydrolysis—unchanged tri-, di-, and mono-glycerides, free fatty acids, and glycerol—are usually isolated by thin-layer or column adsorption chromatography [40,78–89]. If the adsorbent is impreg-

nated with boric acid, diglycerides and monoglycerides are separated into symmetrical 1,3- and 2-isomers and into unsymmetrical 1,2(2,3)- and 1(3)-isomers respectively [65,79]. Each class of lipids is considered quantitatively, and the composition of their acids is determined by gas chromatography [60,78,84,85]. From the content of a fatty acid in the original fat [A] and for one position in the triglycerides, the composition of another position is calculated by means of the formula $3[A] = 2[A_{(1,3)}] + [A]_{(2)}$, and the content of A in the 2-position is $H = 100[A_{(2)}]/3[A]$. [17, 44,80,82,90-92] Acyl migration (Section III, 3) is assessed by decomposition 1(3)-monoglycerides with periodate, and reisolating the 2-monoglycerides [64,83,87,90]. The structure of diglycerides and monoglycerides is determined also by esterifying them with chlorides of fatty acids not present in the given fat, subjecting the artificial triglycerides obtained to further lipase hydrolysis, and examining the cleavage products of the latter [46,68].

Scheme 3



### 3. Determination of Reliability of Enzymic Analysis of Triglycerides

In order to assess the reliability of the results of lipase hydrolysis it is necessary firstly to ascertain whether the rates of elimination of different fatty acids are the same and whether it is a random process, and secondly to know whether positional specificity of hydrolysis is maintained and how it is affected by possible migration of acyl groups [43,60,68,73,93-101].

No great changes in composition of the acids of each class of lipids (Section III, 2) were observed with different times of hydrolysis of ordinary fats having $[Sat_{(2)}] < 10\%$ and $m = 12-18$ carbon atoms in saturated and unsaturated acids; the unchanged triglycerides and combined fraction of free fatty acids and 2-monoglycerides were similar in acid composition to the original fat (provided that the diglyceride content of the mixture was low). Thus there is little fatty-acid specificity in the reaction, while the hydrolysis of 1,3-acyl groups is itself a random process [1, 40,44,52,57,71,99-107].

Acids having $m:e = 20:5$ and $22:6$ in the fats of marine animals, owing to the proximity of their carboxyls to the first double bond and the terminal methyl group (as a consequence of bending of the chain), are detached more slowly than ordinary acids by factors 5–10, and therefore accumulate in the diglycerides and triglycerides of the reaction mixture. The 22:5 acyls, which do not exhibit these steric features, are hydrolysed normally. Lipase hydrolysis has now been replaced by use of a Grignard reagent—methylmagnesium bromide—for the structural analysis of "marine" fats [80,95,107].

On the other hand, short-chain acyls ($m < 12$), owing to the ease of their migration from the 2-position to the 1,3-position in 2-monoglycerides and 1,2(2,3)-diglycerides, are hydrolysed at three times the rate for ordinary acids, so that they accumulate in the monoglycerides. Therefore the results of lipase hydrolysis for milk fat, the fat of Cuphea llavia, the oil of Irvingia gabonensis, and other fats rich in volatile acids are unreliable. The degree of migration of lauric acid, which occupies a position intermediate between volatile and non-volatile acids, has not yet been determined [16,39,53,82,87,93,94, 96,97,100,102-104,106].

Chain branching inhibits the detachment of acyl groups, but the introduction of a hydroxy-group has no effect on the rate of hydrolysis. The dibasic acyls in triglycerides containing azelaic acid were hydrolysed like ordinary acyl groups. Unsaturated acyl groups in pork fat ($[Sat_{(2)}] \simeq 75\%$) were eliminated preferentially, which increased the saturation of the remaining triglycerides and diglycerides. The composition of 2-monoglycerides $[Sat_{(2)}]$ remained unchanged, and therefore calculation of $[A_{(1,3)}]$ (Section III, 2) and the structure of glycerides from $[A_{(2)}]$ and [A] is more correct than the previous calculation of $[A_{(2)}]$ from the variable composition of free fatty acids. Results for the effect of *cis–trans* isomerism on the rate of hydrolysis are contradictory [41,46,47,54,59,98,105].

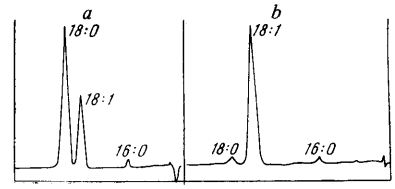



**Figure 3.** Gas–liquid chromatograms of fatty acids: *a*) 1,3-distearo-2-olein; *b*) 2-monoglycerides obtained by lipase hydrolysis of (*a*) [55].

The hydrolysis of synthetic and natural glycerides in the intestine and on a micro-scale *in vitro* for 1–2 min (Fig. 3) corresponds to the chemical equation (Section III, 1) [42,52,57,83]. With increase in the time of reaction the monoglycerides isomerise: they are usually found to contain 10–30% and more of 1(3)-monoglycerides formed by the migration of acyl groups in an aqueous medium at pH 8, and easily converted into glycerol (up to 30–40% by the end of the reaction) [39,66,71]. Isomerisation can be partly eliminated by adding free fatty acids or by using microbial lipases with an acid optimum pH (section III, 4) [41,49,108]. The migration of acyl groups is directed thermodynamically to the 1,3-positions, and its rate is lower than that of detachment. Therefore 2-monoglycerides are free from acyl groups present in the 1,3-positions of the initial triglycerides, and the composition of their acids corresponds to that of the 2-position of these triglycerides [46,47,82].

The positional specificity of lipase is almost absolute [44, 55,105]. Thus hydrolysis of the acylation products of 1(3)- and 2-monoglycerides (Section III, 2) resulted in the elimination of 98% and 2–10% respectively of the original acid, but the composition of the hydrolysis products from derivatives of 1,2(2,3)- and 1,3-diglycerides corresponded to the theoretical value within 5%. Similar results have been obtained in experiments with 1,3-benzylidene-2-oleoylglycerol and 1,3-dihexadecyl-2-oleoylglycerol, where migration of oleate is excluded, while foreign "lipase" (Section III, 2) is inhibited [62,68].

Quantitative estimate were made of migration and specificity of hydrolysis by subjecting 2-[9′,10′-$^3H_2$]oleoyl-1,3-dipalmitoyl-*sn*-glycerol to cleavage at the constant pH = 8 (Table 3 and Fig. 4). The acid composition of the reaction products diverged from the theoretical only as a consequence of the isomerisation of 1,2(2,3)-diglycerides and especially 2-monoglycerides, which increased during incubation; the content of 1-monoglycerides and free oleate in the hydrolysis products did not exceed 3–5%.

Table 3. Hydrolysis of 1,3-dipalmitoyl-2-[9′,10′-$^3H_2$]-oleoylglycerol by pancreatic lipase (mole %).[65]

| Time, min | Free fatty acids in products | Content of $^3H$ (%) in | | | | 1,3-PalOl × 100 / 1,2-PalOl | Moles per 100 moles of 2-MGl | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TGl | DGl | MGl | FFA | | all 1-MGl | 1-MOl | 1-MPal |
| 19 | 31 | 26 | 34 | 37 | 3 | 2 | 23 | 20 | 3 |
| 22 | 50 | 17 | 27 | 52 | 4 | 3 | 23 | 20 | 3 |
| 30 | 60 | 6 | 15 | 74 | 5 | 4 | 38 | 35 | 3 |
| 45 | 60 | 11 | 15 | 66 | 8 | 6 | 43 | 38 | 5 |

The radioactivity of the triglycerides, diglycerides, and monoglycerides obtained from the tritiated triglyceride by hydrolysis with the lipase of the mould Rhizopus arrhizus was respectively 60, 59.9, and 55.5 (in $10^3$ d.p.m. $\mu$ mole$^{-1}$), which indicates slight migration of the tritiated oleoyl from the 2-position[65].

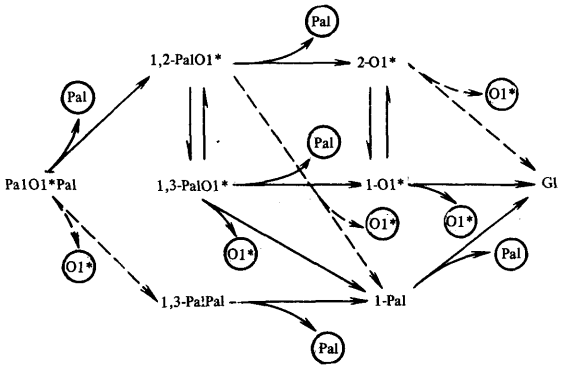



Figure 4. Possible transformations of PalTOlPal on lipase hydrolysis[65], Full lines represent normal hydrolysis of 1,3-acyl groups, and broken lines abnormal hydrolysis of 2-acyl groups. The symbol ⇌ denotes spontaneous acyl migration.

Lipase was used originally for the hydrolysis either of synthetic individual triglycerides or of natural fats of complicated glyceride composition[40,42,44,50]. The results were expressed as the acid composition at 2- and 1,3-positions. The calculation was also based on values of *H* (Section III, 2), other parameters of the positional distribution of individual acids, and the composition of the fat glycerides from the appropriate equations[64,68,93,105]. When $H \simeq 33\%$, a purely statistical distribution of the given acyl group is present[71,82,106]. However, this seldom occurs[63]: the structure of most animal and vegetable fats diverges greatly from the statistical[43,45,83]. The differences in composition between the 1,3- and 2-positions led to the hypothesis that two enzymes, differing in fatty-acid and positional specificity, were responsible for the synthesis of glycerides[35,49,73]. It has now been shown that the best method for the structural analysis of glycerides is a combination of a micromethod of lipase hydrolysis with preliminary chromatography of the fats[1,41,47,54,72,87].

The defects of lipase hydrolysis are the sometimes poor reproducibility of the effects of different lipase preparations containing various impurities, the migration of acyl groups, the re-esterification of free fatty acids due to the transferase activity of lipase on prolonged incubation in the absence of calcium ions, and the absence of stereospecificity of hydrolysis[1,39–42,47,54,67,109–111].

## 4. Use of Other Lipases for the Analysis of Triglycerides

Besides the pancreatic lipase of the pig, the corresponding enzymes from other mammals, and also the lipases of cod, skate, and lobster, which have the same positional specificity, are used in the analysis of fats[39,43,79,112–114]. Lipases of milk and of the fungus Pseudomonas fragi are also specific to the 1,3-positions. The optimum pH for their functioning is 5.6, and therefore acyl migration is less pronounced than with pancreatic lipases[1,44,100,115]. The lipase of Vernonia anthelmintica seeds attacks the 2-position to give 1,3-diglycerides. The enzyme of the fungus Geotrichum candidum is specific, although not absolutely so, to *cis*-9-unsaturated acids, especially to oleic acid, independently of their position (Section IV, 8). The enzyme hardly detaches elaidic acid at all, which demonstrates the stereoselectivity of formation of the enzyme–substrate complex[101,112,116]. The non-specific lipases of the castor-oil plant and of Streptococcus aureus are not used in the analysis of glycerides[54,105]. Hypotheses assuming the participation of plant lipases in the synthesis of triglycerides have now been abandoned[41].

## 5. Structural Analysis of Triglycerides by Mass Spectrometry

Non-enzymic methods for the structural analysis of triglycerides include mass spectrometry (Fig. 5) and also the determination of polymorphism, nuclear magnetic resonance spectroscopy, and infrared spectroscopy (Section V).

The mass spectrum of synthetic 1-myristoyl-3-palmitoyl-2-stearoyl-*sn*-glycerol, obtained on an MS-9 instrument at 170–200°C, exhibited the following peaks: (1) a peak due to the molecular ion $M^+$ at $m/e = 804$; (2) peaks at 523, 551, and 579 formed by the elimination of RCOO groups from $M^+$ (where R = St, Pal, or Myr); (3) peaks due to the ions of these acyl groups at 267, 239, and 211; and (4) peaks due to ions formed by elimination of the fragment $CH_2OCOR$ from $M^+$ (at 509, 537, and 565 respectively). Fig. 5 shows that the intensity due to the ion (peak with $m/e = 509$) resulting from the elimination of $CH_2OCOC_{17}H_{35}$ (the fragment containing the 2-stearate residue from the initial triglyceride) from the $M^+$ ion was

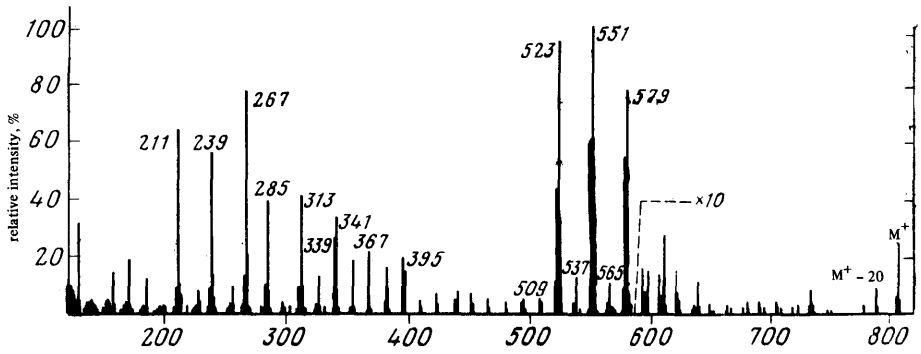



Figure 5. Mass spectrum of 1-myristo-2-stearo-3-palmitin [117].

$CH_2OCO_{13}H_{27}$
|
$CHOCOC_{17}H_{35}$
|
$CH_2OCOC_{15}H_{31}$
$M = 804$

one-third of the intensities of ions with peaks at 537 and 565. Hence the position of an acyl radical in the triglyceride molecule can be established from the relative intensities of the peaks due to $M^+ - CH_2OCOR$. Thus the synthetic triglycerides 2-OlStSt and 1-OlStSt gave different mass spectra. With compounds of molecular weight below 1058 decomposition under the influence of electron impact is not accompanied by thermal decomposition. The mass-spectrometric method is an improvement on lipase hydrolysis with respect to speed and sensitivity of analysis, but as yet it is applicable only to purified individual triglycerides [41,117].

The natural glycerides which have been investigated by mass spectrometry include the optically active triacid triglyceride from the polar fraction of the Chinese oil from Sapium sebiferum, which yields on methanolysis esters of linoleic, linolenic, and deca-2,4-dienoic (10:2) acids. The molecular weight of this glyceride, determined by mass spectrometry, is 920, not the 778 which might have been expected from its fatty-acid composition. The difference of 142 indicates that this compound contains also a residue of a $C_8$ hydroxy-acid. Since the spectrum did not contain a peak due to $M^+ - CH_2OCOR$ (R = 10:2), it could be assumed that the 10:2 acid was not attached directly to the glycerol. These results helped to establish the structure of the optically active triglyceride, as described in detail below (Section IV, 4).

## IV. DETERMINATION OF ABSOLUTE CONFIGURATION OF TRIGLYCERIDES

### 1. Present-day Position of the Problem of the Absolute Configuration of Fats

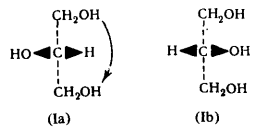
Most of the naturally occurring asymmetric compounds —amino-acids, carbohydrates, terpenes, alkaloids, etc.—are usually enantiomers [35,118]. The structural analysis of triglycerides is often confined to lipase hydrolysis [119], and the stereoisomerism of triglycerides, in contrast to their composition, has been little studied as yet, owing to the difficulty of isolation in a pure form, insufficient development of the preparation of their stereoisomers, and also the fact that modern methods do not usually detect optical activity in the stereoisomers of triglycerides [118].

Optical activity may be expected in asymmetric triglycerides of the type ABC (A = B and A ≠ B) [41], which have now been detected in all natural fats [120]. A racemic mixture of equal quantities of the enantiomers of such glycerides does not possess optical activity. If, however, one enantiomer is completely or partly absent from the mixture, experimental detection of the asymmetric $C_{(2)}$ atom of the glycerol residue is in principle possible [121]. The equivalence or non-equivalence of composition of the acyl groups in positions 1 and 3 in mixtures of optically active glycerides is important for a complete description of the fat and for assessing the reliability of theories of the structure of triglycerides, since many of these theories are based on the hypothesis of a statistical distribution of acyl groups in the 1,3-positions. This problem can also be solved independently of any structural theory [118,121-123].

### 2. Nomenclature of Stereoisomers of Glycerol Derivatives

The glycerol molecule, which is devoid of optical activity, is usually regarded as symmetrical. The problem arises of how to distinguish between carbon atoms 1 and 3, which are differentiated in the exchange reactions of lipids. The Fischer projection formula of glycerol (Ia) in Scheme 4 (where the solid triangles denote bonds directed towards the reader, and the broken lines those directed away from the reader) shows that the halves formed by the plane through the $C_{(2)}$ atom and the HO–H line are not identical, since they cannot be superimposed.

Scheme 4

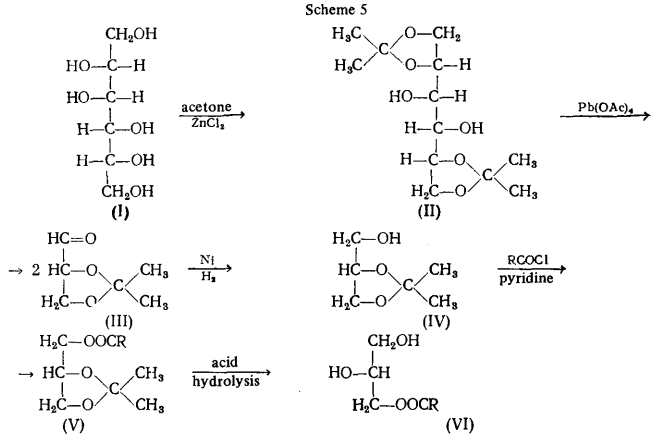



Thus $C_{(2)}$ in glycerol is a *meso*-atom, and glycerol itself has a *meso*-structure, so that it is devoid of rotational symmetry. Indeed, on rotating (Ia) through 180° about an axis perpendicular to the plane of the paper, we obtain (Ib) for glycerol, which cannot be superimposed on (Ia).

The *meso*-structure and the absence of rotational symmetry suggest that the two hydroxymethyl groups will have different fates during exchange reactions with asymmetric reactants (which include all biological reactions), and that they can be distinguished from each other. Thus the biosynthetic replacement of active hydrogen in one of these groups by phosphate, alkyl, acyl, etc. will give a glycerophosphate, batyl alcohol, a 1-monoglyceride, etc. exhibiting optical activity. The steric nomenclature of the carbon atoms of glycerol is therefore important for studying the structure and the biosynthesis of fats [124].

Until recently the Baer–Fischer system was used for denoting the absolute configuration of optically active derivatives of glycerol. In this system a given derivative is assigned to the *D*- or the *L*-series according to which of the two stereoisomers of glyceraldehyde (*D* or *L*) it can be converted into by the least number of reactions. In particular, one stage in such conversion for unsymmetrical mono- and di-glycerides is oxidation of the free primary hydroxymethyl group to an aldehyde group without change in position of the substituents. A non-acylated, phosphate-substituted, radioactively labelled, etc. hydroxymethyl group is always denoted by the Greek letter $\alpha$ and the secondary $CH.OR_{(2)}$ group by the letter $\beta$. This system has several drawbacks for structural and biochemical purposes, since it remains obscure whether $\alpha$ denotes the 1- or the 3-position. Baer and Fischer's nomenclature was modified to the so called "conventional" *D*/*L* system.

Scheme 5




This still used the prefixes *D* and *L*, but the positions of substituents were denoted by the numerals 1, 2, and 3, where "1" denotes acylation of a hydroxymethyl group. This system also has defects: for example, according to this nomenclature the phosphorylation product of an *L*-1,2-diacylglycerol (a phosphatidic acid) can be termed either an *L*-1,2-diacylglycero-3-phosphate or a *D*-2,3-diacylglycero-1-phosphate. Therefore this system has now been replaced by others [124–126].

The more complete (*R*)/(*S*) system is based on the sequence in which radicals of different ranks are arranged about the centre of asymmetry [118]. The rank of a given group depends on its position in the arbitrary hierarchy—OH > COOH > ketone > aldehyde > hydroxymethyl > $CH_3$ > > H, etc. [126] In glycerides longer acyls have priority before shorter acyls, unsaturated before saturated, branched before straight-chain, *cis* before *trans*, etc. A hydroxymethyl group substituted by an acyl of lower rank is numbered "1". In a triglyceride the sequence begins with a group of higher rank—OR at $C_{(2)}$—containing oxygen, and it terminates with the radical of lowest rank—the hydrogen atom at $C_{(2)}$. If the radicals in order of diminishing rank are arranged clockwise in a plane above the $C_{(2)}$ atom viewed from the side opposite to the substituent of lowest rank (the hydrogen atom), the triglyceride belongs to the right-hand or (*R*) configuration; if the opposite is true, it belongs to the left-hand or (*S*) configuration. Thus the optical enantiomers of $\alpha$-laurodipalmitin (Section IV, 3) are denoted in the (*R*)/(*S*) nomenclature as (*S*)-1-laurodipalmitin (I) and (*R*)-1-laurodipalmitin (II) [118]. The advantages of this system are universality and the absence of any ambiguity in the notation. However, the reactions of glycerol derivatives mostly involve only the 1- and 3-ester bonds. As a consequence, the configurational prefixes (*R*) and (*S*) are often interchanged: thus the product of the phosphorylation of an (*S*)-1,2-diacylglycerol is termed an (*R*)-phosphatidic acid. Little use of the (*R*)/(*S*) system is now made in the nomenclature of lipids [124].

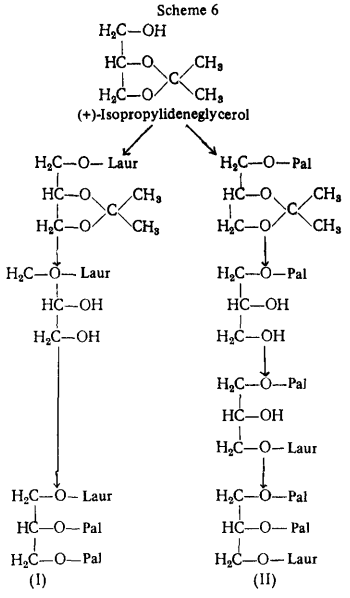
Hirschmann proposed for glycerol derivatives a system of stereospecific numbering with a fixed position of substituents (the *sn*-system), which is now the most widely used for denoting the stereospecificity of triglycerides [124, 126]. If was stipulated that the carbon atoms in the projection formula of glycerol (Ia), in which the substituent of higher rank (the hydroxyl on the *meso*-carbon atom) is always turned to the left, and the hydrogen to the right, should be numbered in the same way as the carbon atoms in *L*-glyceraldehyde, i.e. downwards (I). Thus each primary hydroxymethyl group of glycerol has a definite number in the *sn*-system, and can sometimes be numbered "1" and sometimes "3". Here a triglyceride is termed a 1,2,3-triacyl-*sn*-glycerol, and Baer and Fischer's *D*- and *L*-$\alpha\beta$-diglycerides become 1,2- and 2,3-diacyl-*L*-glycerols respectively. An equilibrium mixture of two enantiomers is denoted in the *sn*-system by the prefix *rac*, e.g. *rac*-1-oleodipalmitoglycerol; if the configuration is unknown the prefix X is used, e.g. X-1-palmitodioleoglycerol. The Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry has now recommended the *sn*-system for general application in denoting the absolute configuration of lipids [124,126].

### 3. Stereospecific Chemical Synthesis of Triglycerides

Owing to the difficulty of isolating stereoisomers from natural fats for the comparison of synthetic with natural enantiomers, methods have been worked out for the stereospecific chemical synthesis of triglycerides [4,120]. Early syntheses were based on the resolution of racemates of intermediate products by means of *D*-tartaric acid, saccharic acid, or strychnine into enantiomers, which were converted into glycerides without racemisation [4]. Nowadays the preparation starts from the optically active natural compound *D*- or *L*-mannitol. In Fischer and Baer's method for the preparation of an *L*-monoglyceride (Scheme 5) 1,2:5,6-di-isopropylidene-*D*-mannitol (II), obtained by treating *D*-mannitol (I) with acetone in the presence of zinc chloride, is converted by oxidative cleavage at the 3,4-hydroxyls with lead tetra-acetate into two molecules of isopropylidene-*D*-glyceraldehyde (III), which is reduced to *D*(+)-isopropylideneglycerol (IV), and this is esterified in the 1-position with an acid chloride to yield the 3-acyl-*L*-glycerol (VI) after the acetone residue

in (V) has been removed by hydrolysis. The 1-acyl-*L*-glycerol is prepared similarly from *L*-mannitol[4]. The stereoisomeric monoglycerides are converted into triglycerides (Section II, 3).

Scheme 6




Scheme 6 shows the synthesis of 1-lauroyl-2,3-dipalmitoyl-*L*-glycerol ((−)(*S*)-1-laurodipalmitin) (I) and 1,2-dipalmitoyl-3-lauroyl-*L*-glycerol ((+)(*R*)-1-laurodipalmitin) (II). Physical methods proved that the products were indeed optical enantiomers[118]. In the stereospecific synthesis of 1,2-dipalmitoyl-3-sorbin the free hydroxyl in (IV) was protected with benzyl, which was removed by hydrogenolysis; the triglyceride obtained was compared with the naturally occurring compound from the oil of S. sebiferum. For the measurement of optical rotation under conditions excluding acyl migration, the 1,2- and 2,3-diglycerides obtained by lipase hydrolysis of the asymmetric triglycerides were acylated with sorboyl chloride or converted into the trimethylsilyl derivatives[120,123]. Triglycerides are synthesised not only from synthetic monoglyceride enantiomers but also from 1,2-diacyl-*L*-glycerols obtained by the action of phospholipase C on egg lecithin[125,127].

## 4. Physical Methods for Determination of Absolute Configuration of Triglycerides

The primary physical method is polarimetry, since many glycerol derivatives—glycerophosphates, batyl alcohol, monoglycerides, diglycerides, trityldiglycerides—exhibit appreciable optical rotation[4,35,118,123]. Among triglycerides only those containing one or two residues of aromatic acids or volatile aliphatic acids have shown rotation: the *L*-forms of these glycerides are laevorotatory, and the *D*-forms dextrorotatory. This coincidence of the configuration and the sign of the rotation is probably fortuitous: the corresponding 1,3-diglycerides do not exhibit such coincidence[118]. The higher fatty acyls, which differ little in chain length and structure, give rise to such weak optical activity in the enantiomeric triglycerides that it cannot be detected by polarimetry even in the ultraviolet. Acylation of optically active *D*(+)-1,2-diolein ($[\alpha]_D^{20} = +1.73°$) in the 3-position at once removes the optical activity[4,34,35,118,120,121,123]. The dispersion of optical rotation at 300–600 nm in synthetic 1,2-dipalmito-3-laurin is 4% of the value in 1-monopalmitin and is opposite in sign of rotation[118]. Thus the absence of rotation in an unsymmetrical triglyceride still does not suggest that it is racemic in composition. Enantiomeric triglycerides, which do not directly exhibit optical rotation even in ultraviolet radiation, are said to be "cryptoactive"[4,34,35,118].

The strong rotation of dolphin fat and of the oils of the plants S. sebiferum and Sebastiana lingustrina (Euphorbiaceae family) is due to the presence of a centre of asymmetry not in the glycerol residue but in the acyl groups[118,120,128]. The polar fraction of the oil from S. sebiferum is an optically active triglyceride ($[\alpha]_D^{20} = -21.9°$). Treatment with lithium tetrahydroaluminate followed by acetic anhydride with the formation of triacetin and the acetates of the aliphatic alcohols shows that this glyceride contains equal quantities of linoleate and linolenate together with 31.5% of deca-2,4-dienoic acid. The optical activity of the glyceride disappeared after hydrogenation, and ethyl decadienoate was inactive. The first paper on this topic attributed the optical activity to the presence of decadienoic acid in the 1- or 3-position of the glyceride, near the potential centre of asymmetry, the $C_{(2)}$ atom of glycerol[128]. However, the value of $[\alpha]_{546}^{20}$ for the synthetic analogue—the 1,2-dipalmitate 3-hexa-2′,4′-dienoate of *sn*-glycerol—was only −3.60°. A later investigation of the glyceride of S. sebiferum by infrared and nuclear magnetic resonance spectroscopy together with mass spectrometry established[129] its true structure as

Linolenoyl.O.$CH_2$.CH(O.Linoleoyl).$CH_2$.O.CO.$[CH_2]_3$.CH:CH*:CH.$CH_2$.
.O.CO.CH:CH.CH:CH.$[CH_2]_4$.$CH_3$.

The decadienoic acid residue was proved to be attached not to the glycerol residue but to the acyl of an optically active $C_8$ hydroxy-allenic acid. Thus the centre of asymmetry C* of the triglyceride occurs not in the glycerol residue but in the aliphatic chain of a $C_8$ acid[128,129].

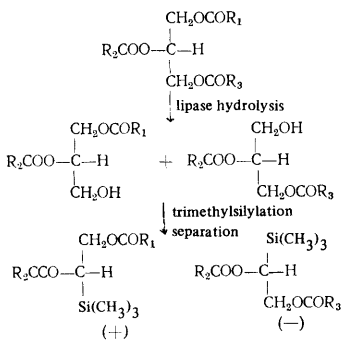
Among natural triglycerides only the *L*-1,2-diacyl-3-acetins—the main components of the seed oils of Euonymus verrucosus, Impatiens edgeworthii, and other plants of the families Celastraceae, Rosaceae, Balsaminaceae, Lardizabalaceae, and Ranunculaceae—have shown measureable optical activity due to asymmetry of the glycerol residue. After hydrogenation the oil contained *L*-1,2-palmitostearo-3-acetin and *L*-1,2-distearo-3-acetin; their mixture had $[\alpha]_{350}^{25°} = 1.9°$, and the main infrared band was located at 8.1 μm, indicating the presence of an acetate group. The optical activity of the stereoisomeric glycerides does not vanish on hydrogenation, storage under ordinary conditions for 6–7 years, or heating to 170°C with the exclusion of acyl migration and oxidation. However, the optical activity was lost after randomisation, which proves that the centre of asymmetry is situated in the glycerol residue of these triglycerides[130–132].

The identification of enantiomers and racemates and establishment of the configuration of cryptoactive triglycerides by physical methods requires the isolation and careful purification of individual glycerides, and these should be examined in the form of crystals. Study of the

piezoelectric excitability of three synthetic triglycerides between electrodes at the a.c. frequency showed that a piezoelectric effect occurred only with enantiomers, but was absent in a racemate. Differences in melting point between the racemate and enantiomers are too small for identification of the latter, and it is therefore necessary to obtain phase diagrams of mixtures of a triglyceride of unknown configuration with each of the corresponding enantiomers [34,118].

It is more convenient to determine the stereoisomerism of triglycerides by X-ray examination. Stereoisomers give the same diffraction pattern, which differs from that due to the racemate. To determine the configuration of 2-OlPalSt of cocoa butter, therefore, the X-ray photographs of a synthetic racemate and of the natural glyceride were compared: the identity of the patterns showed that the natural product was a racemate. If such coincidence had not occurred, it would have been necessary to obtain the X-ray diagram of a mixture of the natural glyceride with one of the synthetic stereoisomers: if the pattern remained unchanged, the cocoa glyceride was idential with this stereoisomer; if not, it was identical with the other stereoisomer [118].

Scheme 7




## 5. Qualitative Detection of Optical Activity of Triglycerides by Chemical Methods

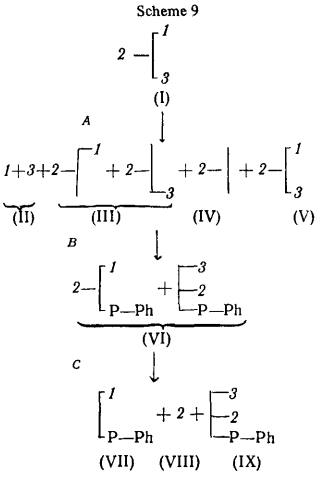
The detection of optical activity e.g. in vegetable SatUnsatUnsat triglycerides, requires their conversion by lipase hydrolysis into a mixture of the 1,2- and 2,3-diglycerides; the diglyceride acetates obtained by acetylation can be oxidised at the double bonds with a solution of potassium permanganate and periodate to give a mixture of AcAzSat and $AcAz_2$ triglycerides. The presence of rotation in this mixture will suggest non-equivalence in the composition of the acids at positions 1 and 3 in the original SatUnsatUnsat glycerides [41].

Appreciable rotation ($[\alpha]_{546}^{20°} = +3.7°$) was observed in synthetic 1,2-dipalmitoyl-3-sorboyl-*L*-glycerol. It was therefore suggested that polarimetry of appropriate derivatives of 1,2- and 2,3-diglycerides—products of the lipase hydrolysis of the fat—could be used to establish the presence of stereoisomers in a fat [41,120]. Polarimetry was applied to the trimethylsilyl ethers of the diglycerides, since they surpassed both sorbic esters and the diglycerides themselves in angle of rotation $[\alpha]$; furthermore, migration of acyl groups was excluded by their use. The standard was the trimethylsilyl ether of *L*-1,2-dipalmitoylglycerol, with $[\alpha] = +4.7°$. The SatSatUnsat and UnsatUnsatSat glycerides of several natural fats were split with lipase, and measurements of the angle of rotation $[\alpha]$ were made on the stereoisomers of trimethylsilyl-sn-1,2- and -2,3-diglycerides of different acid composition obtained by thin-layer chromatography of the silver complexes (Scheme 7). All the triglycerides studied were active, the angles of rotation of enantiomers being opposite in sign; however, all fractions had lower values than the synthetic standard (Scheme 8). In this way we can first establish the absolute configuration of a glyceride or its predominant stereoisomer [123].

Scheme 8

| Fat | Triglycerides | [α] of trimethylsilyl derivative of 1,2-diglycerides | | [α] of trimethylsilyl derivative of 2,3-diglycerides | | Absolute configuration *sn*-system | Absolute configuration Ingold |
|---|---|---|---|---|---|---|---|
| Pork fat | SatSat<br>Unsat | SatSat | +1.6° | UnsatSat | -1.3° | Sat—[Sat, Unsat] | (*R*) |
| Palm oil | Unsat<br>Unsat<br>Sat | UnsatUnsat | +2.5° | SatUnsat | -2.3° | Unsat—[Unsat, Sat] | (*S*) |
| *Myristica malabarica* fat | Unsat<br>Unsat<br>Sat | UnsatUnsat | +0.5° | SatUnsat | -3.9° | Unsat—[Unsat, Sat] | (*S*) |
| Cocoa butter | Unsat<br>Unsat<br>Sat | UnsatUnsat | +1.0° | SatUnsat | -3.2° | Unsat—[Unsat, Sat] | (*S*) |

Scheme 9




## 6. Quantitative Determination with Phospholipase A of the Stereoisomerism of a Natural Mixture of Triglycerides

Brockerhoff's method [125] is based on the steric and positional specificity of lipolytic enzymes (Scheme 9, where *1–3* are acyl groups of fatty acids). Lipase hydrolysis (*A*) of the triglyceride (I) gives the free fatty acids (II), a racemic mixture of *sn*-1,2- and *sn*-2,3-diglycerides (III), a monoglyceride (IV), and unchanged triglyceride (V). The mixture (III) is phosphorylated (*B*) with phenyl phosphorodichloridate to give a mixture of *L*- and *D*-phosphatidylphenols (VI), which is hydrolysed at the 2-position (*C*) by means of stereospecific phospholipase A

(EC 3.1.1.4), to yield *L*-lysophosphatidylphenol (VII), a free fatty acid (VIII), and an unhydrolysed 2,3-diacyl-*L*-glycerol 1-phosphate (IX) (*D*-phosphatidylphenol in Fischer's nomenclature). The composition of the acyls in the 1,2- and 3-positions of the triglycerides can be calculated independently from the acid composition of the initial, intermediate, and final products.

If acyl migration occurs and hydrolysis is specific with respect to fatty acids, the acid composition of the 1,3-positions in the diglycerides will not be identical with that of the original fat [41]. Hydrolysis of *D*-, *L*-, and *DL*-dipalmito-olein by lipase showed that acyl groups *1* and *3* were detached simultaneously and at the same rate, and the diglycerides obtained were optically inactive. The [Pal]/[Ol] ratio in the free fatty acids and in the diglycerides in the reaction mixture (1 : 1 and 3 : 1 respectively) corresponded to the theoretical value [133]. During hydrolysis on a macro-scale stearate was split off more slowly than other acyl groups [68]. To ensure statistical hydrolysis hexane was added to the medium (Section III, 2); this weakened the van der Waals forces that retained selectively the longer and less curved acyl groups on the surface of the fat micelle [9,125]. The detachment of polyunsaturated acids having $m = 20–22$ lagged behind even with hexane, so that they accumulated in the diglycerides. Therefore "marine" fats and also pork fat and maize oil were deactivated with a Grignard reagent (0.06–0.12 M $CH_3MgBr$ in ether): symmetrical monoglycerides and diglycerides showed considerable changes in composition owing to migration of the acyl groups, but 1,2(2,3)-diglycerides corresponded in composition to the original fat, and could be used for stereospecific analysis [9,41,107,134–136]. The composition of the acids of 1,2(2,3)-diglycerides, obtained by hydrolysis on a macro-scale [125,137], differed markedly, by an increased content of saturated acyl groups, from that calculated theoretically. A micro-method of hydrolysis gave better agreement [47,134]. It is possible that statistical hydrolysis is ensured by a diminished calcium-ion level and an increased enzyme content in the reaction mixture [41].

In Brockerhoff's method the diglycerides obtained by hydrolysis (yield 20–30%) were separated into 1,2(2,3)- and 1,3-diglycerides by chromatography on silica gel impregnated with boric acid; in the presence of phenyl phosphorodichloridate, ether, and pyridine at 0°C the 1,2(2,3)-diglycerides were converted into phosphatidylphenols, whose triethylammonium salts were hydrolysed with phospholipase A in ether at pH 7.5 in the presence of calcium chloride. The reaction products were separated by chromatography on an adsorbent impregnated with boric acid, and the acid composition of each fraction was determined by gas chromatography [9,125,135,136]. The compositions of positions 1, 2, and 3 are reliable only if the analytical results show the following equalities: (I) = (V), (III) = (VI), and (IV) = (VIII) (Scheme 9); these indicate that hydrolysis by lipase and by phospholipase has occurred statistically and without isomerisation. The compositions of positions 1 (VII) and 2 (IV) and (VIII) are determined directly; the acid content in the 3-position is calculated in two ways, as either 3[(I) – (IV) – (VII)] or 2[(IX) – (IV)]. An absolute divergence of 4% is acceptable when [A] ≥ 40%, not more than 3% with 10–35%, and at most 2% when [A] < 10%; the error in the calculation does not usually exceed ±2%, and only with difficultly hydrolysable saturated fats does it increase to 8%. [9,10,119,125,136] However, if the results show good reproducibility, only fractions (I), (IV), (VII), and (IX) are analysed [9]. Brockerhoff's method has been tested on synthetic and semi-synthetic triglycerides [125].

Direct analysis of the composition at the 3-position was accomplished by converting the 1,3-diglycerides, obtained by deacylating the fat by means of a Grignard reagent, into *L*-2-phosphatidylphenols and splitting the latter at position 1 with phospholipase A. The resulting lysophosphatides corresponded in acid composition to the 3-position of the initial fat [135].

### 7. Determination of Absolute Configuration of Individual Triglycerides

In all the fats studied, positions 1 and 3 differ in composition. This proves indirectly the presence of stereoisomeric triglycerides, but does not indicate their nature [123]. However, even identity of composition at positions 1 and 3 in a mixture of triglycerides would not necessarily prove that all the glycerides in the fat were racemates. Thus the ratio of palmitoyl to stearoyl at positions 1 and 3 is unity for the equimolecular mixture—(*R*)-PalOlSt + (*S*)-PalPalSt + (*R*)-PalOlSt + (*S*)-PalPalSt + (*S*)-PalOlSt + (*R*)-PalPalSt—although this mixture contains one-third of the racemate 2-OlPalSt plus one-third of the racemate 2-PalPalSt plus one-sixth of the enantiomer (*R*)-PalOlSt plus one-sixth of the enantiomer (*S*)-PalPalSt. Therefore the absolute configuration can be assessed only by analysing individual glycerides or simple mixtures in which a certain acyl group (e.g. palmitoyl in (*S*)-PalOlSt + StOlSt) is present only in one of the outer positions in one of the glycerides [118].

The problem of analysing the configuration of mixtures and individual types of diacid ($A_2B$) triglycerides has now been solved, at least in principle: metamers having the same *m* and *e* but different numbers of saturated acyls (e.g. StStLinoleoyl and StOlOl) can be separated; the proportions of stereoisomers in $A_2B$ are easily found from the content of the acid B at positions 1, 2, and 3 in the mixture AAB + ABA + BAA. Establishment of the configuration of triacid glycerides—i.e. the proportions of the six possible stereoisomers ABC, ACB, BAC, BCA, CAB, and CBA—is a more complicated matter. From the composition of the acids at positions 1, 2, and 3 it is possible to find the proportions of any pairs of isomers, e.g. pairs with respect to the content of acid A:[122]

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (I) | AA | BC | BC | (II) | AB | AC | BC |
| | BC | AA | CB | | BA | CA | CB |
| | CB | CB | AA | | PP | PP | PP |
| | | | | | *x* | *y* | *z* |

An additional parameter is necessary for calculation of the quantity of each stereoisomer in the mixture (I), and can be obtained by separating the intermediate products in the analysis according to the acid composition: the glyceride ABC is hydrolysed with lipase, and 1,2(2,3)-diglycerides are isolated; the 1,2-diglycerides are converted into phosphatidic acids by means of stereospecific diglyceride kinase, and the acid composition of each position is found by Brockerhoff's method modified by first separating the phosphatidic acids into pairs of stereoisomers according to acid composition (II). The proportions of the pairs *x*, *y*, and *z* are equal to the proportions of the acids C, B, and A respectively in the 3-position. The proportions of isomers within a pair are determined by hydrolysis with phospholipase A [138].

8. Use of Other Enzymes than Phospholipase A for Analysis of Stereoisomerism of Triglycerides

If the glyceride ABC contains oleate, the additional parameter for estimating the stereoisomers can be obtained by means of the lipase of G. candidum (Section III, 4) together with Brockerhoff analysis; the 1,2(2,3)-diglycerides obtained by the elimination of oleate from the 1(3)-positions are converted into phosphatidylphenols, and the ratio of stereoisomers in a pair is determined by hydrolysis with phospholipase A [139].

Other stereospecific enzymes of lipid metabolism are suitable for the analysis of stereoisomerism. A racemic mixture of phosphatidylphenols can be resolved by hydrolysis with phospholipase C, which is specific to the $L$-isomer [9]. The triglycerides of rat liver have been analysed by means of the diglyceride kinase of Escherichia coli, which converts 1,2-diglycerides, obtained by lipase hydrolysis, 25 times as rapidly as 2,3-diglycerides into phosphatidic acids [137,140,141]. The contents of each acid at positions 1, 2, and 3 were calculated by means of the formulae

$$[A]_1 = (2[A]_{Phtd} - [A]_{MGl}) : 3;$$
$$[A]_2 = ([A]_{MGl}) : 3;$$
$$[A]_3 = [A]_{TGl} - (2[A]_{Phtd}) : 3,$$

where $[A]_{TGL}$, $[A]_{MGl}$, and $[A]_{Phtd}$ are the contents of acid A in the initial triglycerides and in the 2-monoglycerides and phosphatidic acids obtained from them. These equations hold only if the fatty-acid composition of the 1,2(2,3)-diglycerides obtained by lipase hydrolysis corresponds to the calculated composition (Section IV, 6) and substitution of $[A]_{1,2(2,3)}$ for $[A]_{1,2}$ in the equation gives the same acid composition in 1- and 3-positions. The differences observed indicate the minimum error of the method. These differences are often considerable, which is evidence of the fatty-acid selectivity of lipase hydrolysis. It is therefore necessary in each experiment to ensure agreement between experimental and theoretical acid compositions [134].

## V. POLYMORPHISM OF TRIGLYCERIDES

### 1. Lattice Structure and Phase Transitions of Triglycerides

The phenomenon of polymorphism was discovered as early as 1821 by Mitscherlich. The various forms of a given compound differ appreciably in crystal structure, lattice parameters, melting point, density, and other properties, but have the same composition and chemical structure and, unlike position isomers, give an identical liquid or gaseous phase. The crystalline modifications of carbon—graphite and diamond—provide an inorganic example of polymorphic forms. Organic compounds also exhibit polymorphism, especially long-chain aliphatic compounds, including natural glycerides. Before the 1930s the triple melting points of a triglyceride, discovered more than a century earlier, were attributed to the presence of a series of position isomers; only Malkin proved that the melting anomalies were due to differences in the crystal lattice. When a solidified melt (the polymorphic $\alpha$-form) of a simple $Sat_3$ glyceride is heated slowly, it melts, and then solidifies again with the evolution of heat, to form the stable $\beta$-form or an intermediate $\beta'$-form; the latter appears when the melt is maintained at 1–2 deg above the melting point of the $\alpha$-form. The direction of these transitions, which may not be accompanied by fusion, is from less stable modifications, whose formation requires a minimum change in free energy of the lattice, to thermodynamically more stable polymorphic forms having little free energy. The principal $\alpha$-, $\beta$-, and $\beta'$-forms of triglycerides differing in composition and structure have similar properties, since the acyl chains play the decisive part in the polymorphism. However, new, additional polymorphic forms are continually being found in the huge variety of natural fats, and therefore a classification and a nomenclature have not yet been established for the latter. The direction and the products of polymorphic transitions depend on the purity of the material, the presence and nature of a crystal seed, the solvent, the pressure, the temperature, its rate of change, etc. Thus the crystalline, thermal, X-ray diffraction, and other properties of a triglyceride under given environmental conditions can be assessed only after establishment of the polymorphic form in which the compound is then present. Several reviews have appeared on the polymorphism of triglycerides [4,142-154].

X-Ray diffraction indicates that aliphatic chains are zigzag structures with rectilinear axes, in which the angle between the carbon–carbon bonds is 109°28', and the increase in the length of an acyl residue accompanying expansion of the latter by a methylene group is $a = 1.28$ Å. [4] If the temperature is not sufficiently low, close approach of chains to one another is difficult, and the triglycerides remain liquid, without an ordered structure. As the acyls come closer together on cooling, van der Waals forces of attraction become effective between carbon atoms of adjacent chains: i.e. the triglycerides crystallise [146,147].

Crystals formed by identical aliphatic molecules, whose ends differ in polarity, contain a mainly parallel arrangement of chains forming a kind of "palisade" and usually a crystalline double layer by means of the hydrogen bonds of the polar groups. A vivid model of this arrangement would be two bundles of very large numbers of matches, where the heads represent methyl groups and the free ends the polar groups: one bundle is placed with the heads in a plane, and the other with the free ends on the first bundle. Thus the double layers of molecules are bounded by non-polar "methyl" planes, the distance between which is termed the long spacing $L$ (in Å). The value of $L$ found from X-ray examination enables us to determine the number of chains constituting the long spacing [4].

If the double layers $2m$ long are perpendicular to the methyl planes, $L = 2m$. Layers having $L$ which are multiples of $3m$ or $4m$ occur in crystals of mixed glycerides. Mostly, however, $m < L < 2m$, and the angle of tilt between the axes of the chains and the planes $\beta_X < 90°$. The variation in $\beta_X$ between different crystalline forms is a principal cause of the polymorphism of triglycerides: decrease in $\beta_X$ strengthens contact between their more polar ester groups, and hence improves the stability of the polymorphic forms [143,146,149]. The value of $L$ for a given form varies with the acid composition of the triglycerides. The hypothesis that decrease in $L$ is due, not to tilting of the chains, but to erratic secondary bends [149], has not yet been proved experimentally.

The parallel chains in triglycerides form a unit cell—a right prism or an oblique prism—whose width, termed the short spacing $S$, is determined by the mode of packing of chains in the crystal lattice. This is the other main reason for the polymorphism of glycerides. In contrast

to $L$, values of $S$ for a given form are almost constant and independent of $m$.[143,147,154]

Fig. 6 shows a model consisting of two pairs of chains in the unit cell of stearic acid[4]. A triglyceride unit cell containing two molecules is more complicated: since the axis of one acyl forms an angle of 180° with those of the other two acyl groups (Fig. 6), each chain in the lattice coincides with the long axis of the four-sided prism formed by the other four chains (Fig. 7)[149].

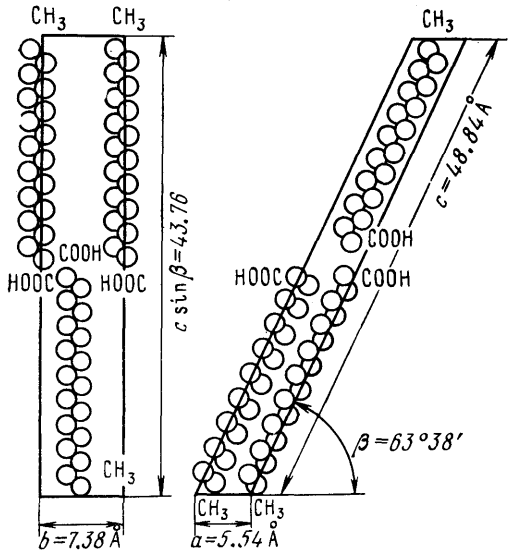



Figure 6. Unit cell of crystal of polymorphic $\beta$-form of stearic acid[4]: $a$ and $b$ are short interplanar spacings; $c \sin \beta_X$ is the long interplanar spacing.

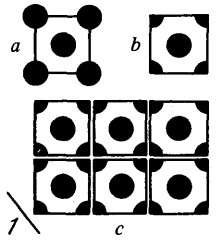



Figure 7. Unit cell of triglycerides[149]: *a*) cross-section; *b*) cross-section with two molecules (or double molecules) belonging to the cell; *c*) cross-section through crystal.

Fig. 8 illustrates possible configurations of the molecules in triglyceride crystals. It was assumed initially that the fork configuration predominates, and later that a chair configuration will give denser packing. X-Ray examination of a single crystal of the $\beta$-form of trilaurin has now shown (Fig. 9) that a rod configuration gives the most economical packing with the smallest free energy, without contradicting the principles of symmetry[143,144].

The crystal is seen to consist of $2m$ layers of rodlike molecules in triclinic packing: two acyl groups are located in a straight line, while the third (it is not yet known which—whether the middle or one of the outer acyl groups) is located at their side and parallel to them. Good agreement is evident between values of the angle $\beta_X$ found from the X-ray photograph and calculated from $L$ (62°7′ and 61°35′ respectively). The rod form ensures also the formation of $3m$ and $4m$ layers in the crystal (Fig. 8)[143,149]. The shape of the molecules in the other main modifications ($\alpha$ and $\beta'$) has not yet been ascertained[151].

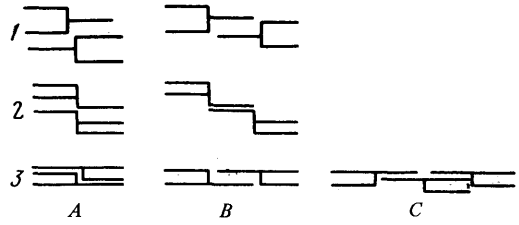



Figure 8. Possible configurations of triglycerides in crystals: 1) fork; 2) chair; 3) rod. Long spacing multiple of: *A*) $2m$; *B*) $3m$; *C*) $4m$.

Phase transitions in heterogeneous systems may be either enantiotropic, i.e. occur reversibly in both directions without melting as the temperature is varied, or monotropic, these being irreversible processes accompanied by loss of free energy in the direction of greater stability, and regeneration of the unstable from the stable form requires intermediate fusion. The phase transitions of triglycerides are mainly monotropic[143].

Pure triglyceride preparations, uncontaminated by homologues of closely similar composition or structure, are necessary in order to obtain sharp polymorphic transitions and forms having definite properties. Thus low-resolution nuclear magnetic resonance spectra indicate the absence of a liquid phase below the melting point in pure specimens of trilaurin and tristearin, but this phase appeared long before melting in contaminated crystals of the $\beta$-forms of OlPalPal, PalOlPal, PalOlOl, and StOlSt.[152,161]

In supercooled melts the dependence of the logarithm of the viscosity on the reciprocal of the absolute temperature usually shows a break in the curves immediately below the melting point of the $\beta$-form, indicating intensification of aggregation of the glycerides by parallel packing. On the other hand, the crystalline structure of a triglyceride is partly preserved immediately after melting: on rapid cooling, this melt immediately crystallises. After the partly ordered structure of the liquid phase has been destroyed by being kept at a temperature 20 deg above the melting point for 30 min, crystals form slowly[153,160,164]. When tripalmitin is crystallised from a solvent, the properties of the resulting crystals of the $\beta$-form depend on the nature of the latter[157].

Table 4 indicates the conditions under which the main polymorphic forms of simple $Sat_3$ triglycerides are obtained[143,146], together with several methods for investigating the polymorphism. The early methods involved determining melting points in capillary and differential

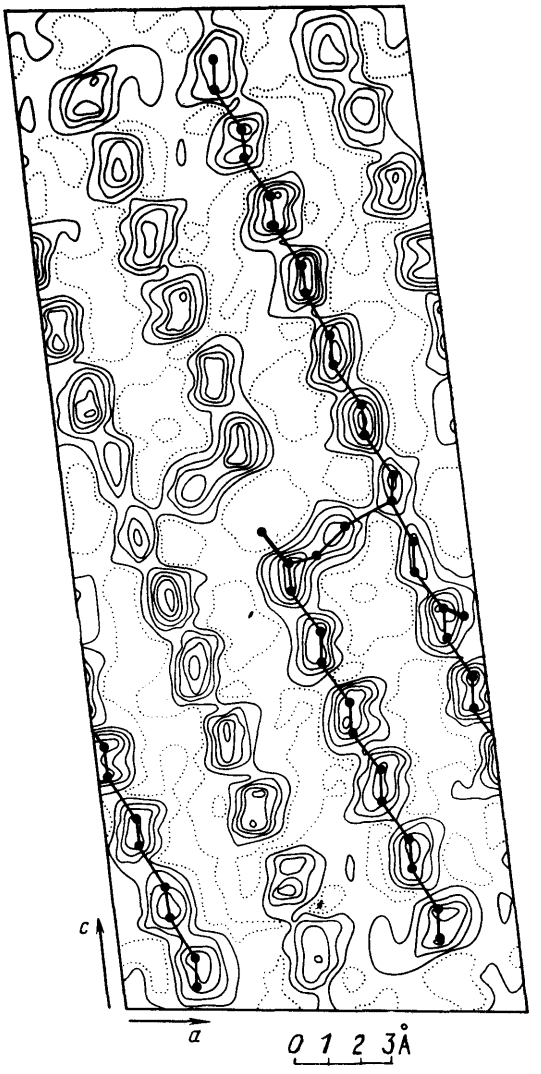



Figure 9. X-ray electron density diagram of polymorphic $\beta_L$ form of trilaurin[143].

thermal analysis, consisting in recording continuously the evolution of heat during solidification, or its absorption during fusion, from the curves for the difference in temperature between the surrounding medium and a sample of the triglyceride during cooling or heating respectively[4,5,33,155-163]. Dilatometry records the increase in specific volume $V_S$ (in ml $g^{-1}$) of the triglyceride at a phase transition point—the melting point—and also the equal but opposite change (contraction) at the freezing point. Both processes are recorded as functions of temperature[148,156,158]. Heating the $\alpha$- and $\beta$-forms of tripalmitin in a microdilatometer at a rate of 6.4 deg $min^{-1}$ (Fig. 10) led to the discovery that the differences between initial and final specific volumes when the $\beta$-form melts are due to the formation of an intermediate $\beta'$-form when the melt sets. The setting of trilaurin and tristearin melts led directly to the metastable $\alpha$-form, whose stability increased with increase in $m$; heating the latter resulted in appearance of the $\beta$-form[157].

Polymorphic transitions governed by temperature can be detected by periodic determination of the number and magnitude of $L$ and $S$ characteristic of particular forms of a given glyceride, and also from the X-ray diffraction diagrams of the powdered crystals or a large single crystal (Fig. 9)[154,159,164,165]. Changes in the packing of chains on passing from an unstable to a stable polymorphic form can be detected by infrared spectroscopy (Fig. 11) from the characteristic skeletal vibrations at 2–15 $\mu$m (5000–600 $cm^{-1}$).[147,149,155,159,162] Finally, low-resolution nuclear magnetic resonance spectroscopy evaluates the rotational mobility of the acyls in a triglyceride lattice, detects the thermal $\alpha \rightarrow \beta$ transition in tristearin, and enables small quantities of liquid phase in triglyceride crystals to be determined to ±2%. This method is more effective than dilatometry, which gives only the relative content of the solid phase[161,164].

Table 4. Properties of main polymorphic forms of simple saturated triglycerides*.

| Notation of form in nomenclature of: | | | |
|---|---|---|---|
| Malkin | Vitreous | $\alpha_M$ | $\beta_M$ |
| Lutton | $a_L$** | $\beta'_L$** | $\beta_L$** |
| Gunstone | $V$ | $U$ | $T$ |
| Angle $\beta_X$, degrees | 90 | 68 | 64 |
| Alternation of m.p. of "even" and "odd" homologues (°C) | absent(54.5) | absent(65.0) | absent (71.5) |
| Short spacing, Å | 4.15 | 3.8 4.2 | 4.6 |
| Value of $c$ (Å) for calculating long spacing from Gunstone's eqn. with even $m$ and | | | |
| $L$ multiple of $2m$*** | 4.9 | 4.3 | 3.7 |
| $L$ multiple of $3m$ | — | — | 8.4 |
| Packing of chains | hexagonal | orthorhombic | triclinic |
| Main absorption bands of group V (Table 5), $cm^{-1}$ | 720 | 727, 719 (doublet) | 717 |

*Corresponding data for tristearin given in parentheses.
**Main methods of preparation: $\alpha_L$ by freezing a melt below 0°C; $\beta'_L$ by freezing a melt at 1–2 deg above the melting point of the $\alpha_L$ form; and $\beta_L$ by crystallisation from polar solvents or by slowly heating crystals of the $\alpha_L$ form above its melting point.
***In Gunstone's equation $m_A = 0$.

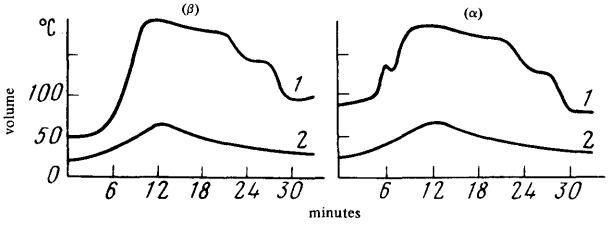



Figure 10. Curves for polymorphic forms (stable $\beta$ and metastable $\alpha$) of tripalmitin[156]: 1) dilatometric; 2) temperature.

## 2. Polymorphic Forms of Trisaturated Triglycerides

The polymorphic forms and several of their properties are given in Table 4;[143,146] fuller information will be found in Markley's monograph[4]. There is as yet no generally

accepted nomenclature for the forms (Table 4). Malkin's early nomenclature, which distinguishes four forms of a given glyceride—vitreous, $\alpha$, $\beta'$, and $\beta$—in order of rising melting point, is defective, since different forms differing in crystalline structure may have closely similar or even identical melting points [4,145,149]. According to Lutton no vitreous form devoid of crystalline structure actually exists, and there are only three main forms—$\alpha$, $\beta'$, and $\beta$-characterised by the spacing $S$ (Table 4).[147,154,165,166] To avoid confusion the nomenclature used for a given form is denoted by a subscript indicating the author ($\alpha_M$, $\alpha_L$, $\beta'_L$, etc.), and the number of chains in $L$ is denoted by a numeral following the subscript ($\beta'_L$-2, $\beta'_L$-3, etc.).[144,146,151,159]

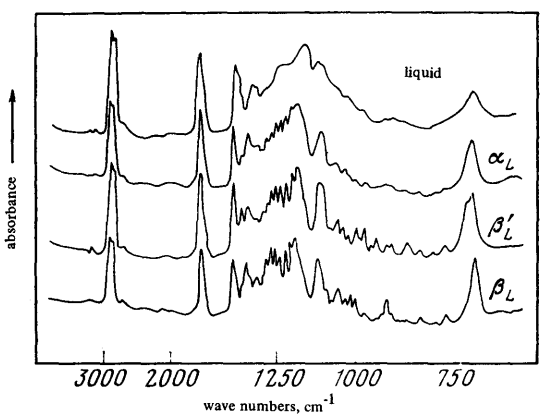



Figure 11. Infrared spectra of polymorphic forms of tristearin[143].

The number and the properties of individual polymorphic forms of triglycerides have been determined also by infrared spectroscopy. The characteristics of the infrared spectra of melts or solutions of simple $Sat_3$ glycerides are given in Table 5.[33,142,155,159] Solid glycerides are characterised by the intensity and the number of vibrational bands in group V; three types of infrared spectra, corresponding to Lutton's principal modifications, have been found from these sets of bands (Table 4).

In the $\alpha_L$ modification (Table 4), which is formed from the melt, the chains are 5 Å apart, and rotate freely about their vertical axis. The numerous bands due to the $\alpha_L$ form at 1250 cm$^{-1}$ prove that it is crystalline, not vitreous as assumed by Malkin. In the glycerides trilaurin and above, which have a stable $\alpha_L$ form, the number of these bands is $\frac{1}{2}m$, which enables the length of the acyls in the glycerides to be determined from the infrared spectrum[143].

The properties of the $\beta'_L$ form (Table 4) indicate the usual orthorhombic packing of two types of tilted chains, in mutually perpendicular planes. Formation of this modification of tristearin has been observed on crystallisation from paraffin oil[143,152].

Finally, the $\beta_L$ form (Table 4) corresponds to triclinic packing, in which all planes of the zigzag aliphatic chains are mutually parallel. The chains themselves are more stable and are packed more densely than in the $\alpha_L$ form. In tristearin the amplitude of rotational oscillations of the chains about the vertical axis does not exceed 17°.[143]

The nomenclatures of Malkin and of Lutton do not enable triglycerides to be compared with other aliphatic compounds with respect to the properties of their polymorphic forms. In Gunstone's nomenclature[146] the polymorphic forms of long-chain saturated compounds are divided into five groups according to the angle $\beta_X$, triglycerides occurring in the first three groups $V$, $U$, and $T$ (Table 4). The value of $L$ in crystals of mono-, di-, and tri-acid $Sat_3$ triglycerides ABC (the acyl group B always occupies the 2-position), being a linear function of the chain length $m$, can be calculated by means of the equation $L = 1.28 \sin \beta_X (m_A + mB + mC) + c$, where $m_A$, $mB$, and $mC$ are the numbers of carbon atoms in the acyls A, B, and C, while $c$ is the constant increment in $L$ (in Å) representing the length of a glycerol residue and the zone of contact of the ester groups. Table 4 gives values of $c$ for $2m$ and $3m$ layers of simple and mixed triglycerides containing acyl groups A, B, and C of different lengths. For a double ($2m$) layer $m_A$ in Gunstone's equation is regarded as zero. Good agreement was found between calculated values of $L$ and those obtained from X-ray data[146].

Table 5. Main absorption bands in the infrared spectrum of simple trisaturated triglycerides.

| Group of vibrations | I | II | III | IV | V |
|---|---|---|---|---|---|
| Absorption bands, cm$^{-1}$ | 3030—2967 | 1751—1733 | 1464—1453, 1383—1361 | 1179—1166 | 1261—1250, 730—717 |
| Vibrating group | C—H | C=O in COOR | $CH_2$ | C—O | $CH_2$ |
| Mode of vibration | stretching | stretching | scissor deformation | stretching | rocking deformation |

Gunstone's numenclature showed for the first time that each polymorphic form is characterised by a definite angle of tilt, but it is unsuitable for calculations on unsaturated glycerides and does not allow for additional polymorphic forms of simple $Sat_3$ glycerides having the same value of $\beta_X$ as the principal modifications[151]. The sub-$\alpha_L$ form is one such additional modification: it is produced without fusion from the $\alpha_L$ form of tristearin at −50°C, is converted reversibly into the $\alpha_L$ form, and is identical with the $\beta'_L$ form in all properties other than the angle $\beta_X = 90°$. Crystallisation from a solvent or heating of the $\alpha_L$ form sometimes results in the appearance of a series of stable $\beta_L$ forms, identical in diffraction pattern but differing in $\beta_X$. In contrast to "even" triglycerides, "odd" acids are stable not in the $\beta_L$ but in the $\beta'_L$ form[142,143,152,159].

Attempts to allow for the additional forms were made by Hoerr and by Larsson. Hoerr's nomenclature allows for four modifications—$\alpha$, $\beta'$, intermediate, and $\beta$—in order of rising melting point. The intermediate modification, which appears when the $\alpha$ form is heated, has since proved to be a mixture of equal quantities of orthorhombic and triclinic forms[146,148]. According to Larsson, (1) forms having a single value of $S = 4.15$ Å are denoted $\alpha$; (2) forms having two values of $S$ (4.2 and 3.8 Å) and a doublet at 720 cm$^{-1}$ are denoted $\beta'$; and the symbol $\beta$ is applied to all forms which do not correspond to criteria (1) and (2), the presence of several such forms of a given glyceride being indicated by $\beta_1$, $\beta_2$, etc. in order of decreasing melting point. According to Larsson all planes of the zigzag chains are parallel in $\beta$ forms, mutually perpendicular in $\beta'$ forms, and show no definite regularity of arrangement in $\alpha$ forms[151].

Lutton's nomenclature, in spite of its defects, has remained the most acceptable up to the present. It comprises three main forms of simple $Sat_3$ glycerides—unstable $\alpha_L$, intermediate $\beta'_L$, and stable $\beta_L$—which must be regarded as sets of crystalline modifications that have closely similar properties but are not identical. Further research is required to achieve a complete classification [143].

Table 6. Polymorphic forms of some di- and tri-acid saturated triglycerides.

| TGl | $\Delta m$ | Principal forms | | | Additional forms | Infrared spectrum resembles | Remarks |
|---|---|---|---|---|---|---|---|
| | | $\alpha_L$-2 | $\beta'_L$-2 | $\beta_L$-2 | | | |
| PalStSt | +2 | + | + | + | $\beta_L$-3 | StStSt | (*a*) |
| StPalPal | +2 | + | + | + | ditto | PalPalPal | |
| StPalSt | −2 | + | − | + | » | StStSt | (*b*) |
| PalMyrPal | −2 | + | − | + | » | PalPalPal | (*b*) |
| PalStPal | +2 | + | + | − | » | PalPalPal | (*c*) |
| MyrPalMyr | +2 | + | + | − | » | MyrMyrMyr | (*c*) |
| StMyrSt | −4 | + | + | + | $\beta'_L$-4 | StStSt | (*d*) |
| StLaurLaur | −6 | + | + | − | $\beta'_L$-3, $\beta_L$-3 | | |

(*a*) $\beta'_L$ and $\beta_L$ forms equally stable.
(*b*) $\beta_L$ form the most stable.
(*c*) $\beta'_L$ form the most stable; $\beta_L$ form difficult to detect.
(*d*) $\beta_L$ and $\beta'_L$ forms have similar values of $L$.

The polymorphism of diacid and triacid $Sat_3$ glycerides (Table 6) is extremely individual, depending on the difference $\Delta m$ between the lengths of the acyls in the 1,3- and 2-positions, the symmetry of the structure, and the presence of an acyl of a lower fatty acid in the 2-position. Sets of polymorphic forms are analogous in homologous series of triglycerides having a certain value of $+\Delta m$ or $-\Delta m$, while symmetrical and unsymmetrical triglycerides of the same composition have identical values of $L$ for $2m$ and $3m$ layers, in good agreement with Gunstone's calculated value (Table 4). In different triglycerides the $\beta_L$, $\beta'_L$, or $\alpha_L$ form is the most stable. Thus in the glycerides of behenic acid the $\beta'_L$ forms, not the $\beta_L$ forms, are stable. Those which are difficult to detect are formed only in the presence of crystals of succinic acid. Mixed glycerides containing two acyls of any acid are almost identical with simple glycerides of the same acid in the number of infrared bands of the $\alpha_L$ form in the region of 1250 $cm^{-1}$. [142,143,159,166,167] The complexity of the polymorphism increases with the absolute magnitude of $\Delta m$ (Table 6). An important part in the polymorphism of mixed glycerides containing acyls of volatile acids is played by the carbonyl groups of the latter, with the formation of unique polymorphic forms [4,146,154].

### 3. Polymorphic Forms of Other Types of Triglycerides

The less studied simple $UnSat_3$ triglycerides in general resemble one another and the corresponding $Sat_3$ glycerides in the set of polymorphic forms and their properties. Sometimes additional forms occur, e.g. $\beta_L$-3 in *cis*-unsaturated triglycerides, which are characterised by bands at 690 and 1660 $cm^{-1}$ due to scissor vibrations of the hydrogen atoms in the *cis*-bond. Triolein resembles tristearin in the main infrared absorption bands of the polymorphic forms at 723 ($\alpha_L$), 729 and 722 ($\beta'_L$), and 722 ($\beta_L$) $cm^{-1}$. When the *cis*-bond is replaced by a *trans*-bond, a band appears at 963 $cm^{-1}$ in all the modifications, and the similarity of the polymorphism with that of $Sat_3$ glycerides of the same length is still more pronounced. The number of $Unsat_3$ bands at 1250 $cm^{-1}$ is $\frac{1}{2}m$, as with $Sat_3$ (Section V, 2) [142,144]. Thus unsaturation in simple triglycerides has little effect on polymorphism [4].

Table 7. Properties of polymorphic forms of triglycerides of cocoa butter, 2-OlPalSt and 2-OlStSt.

| Form | sub-$\alpha_L$-3 | $\alpha_L$-2 | $\beta'_L$-2 | $\beta_L$-3 (V) | $\beta_L$-3 (VI) |
|---|---|---|---|---|---|
| Melting point, °C | 17 | 23 | 27 | 33 | 36 |
| Short spacing, A | 3.8and4.2 | 4.2 | 3.8and4.2 | 4.6 | 4.6 |

Triglycerides of the type SatUnsatSat, constituting ~ 80% of the economically important oil of cocoa beans, have a large set of polymorphic forms. The polymorphism of this oil has been well studied, since it was the chief method of determining the structure of glycerides before the appearance of lipase hydrolysis [168-171]. Crystalline fractions of the oil are identical with a mixture of synthetic PalOlSt, StOlSt, and PalOlPal, while the PalOlSt isolated from the oil coincides with the synthetic triglyceride in melting point, heating curves, dilatometry, diffraction patterns, and infrared spectra of their individual polymorphic forms before and after hydrogenation (Table 7) [33,142]. A mixture of PalOlSt and StOlSt gives three polymorphic forms and differs from either component in rate of conversion of unstable forms, whereas a mixture of StOlSt and StStOl (1 : 3) may give a new crystalline form not present in the initial components [150, 162,165]. Comparison of the polymorphism of StOlSt and StStOl showed no parallelism with the corresponding saturated isomers (Table 6). [168] In homologous series PalPalOl is analogous to StStOl in its polymorphism, and PalOlPal is analogous to StOlSt; in the unsymmetrical $SatUnsat_2$ glycerides StOlOl and PalOlOl, as also with StStOl and PalPalOl, the $\beta'_L$-3 form is stable [170]. Nuclear magnetic resonance spectroscopy indicates that the oleate chains in StStOl are less densely packed than are the saturated acyls, and rotate more freely about the axis [4,5, 143,144].

Polymorphism has been most thoroughly studied, apart from StStOl, on the SatSatUnsat glycerides of pork fat [89]. The main component is identical in the properties of its polymorphic forms with OlPalSt [33,150]. Coarse-grained crystals of the $\beta'_L$-3 form of this triglyceride appear when pork fat is stored at 27°C, and impair the appearance of the product [172], a phenomenon which is eliminated by transesterification (Section II, 1). [163] In contrast to OlPalSt, StStOl, and PalOlOl, the symmetrical glycerides of pork fat OlStOl and OlPalOl give the forms $\alpha_L$, $\beta'_L$-2, and $\beta_L$-3, the last of which is stable [170].

The $\beta_L$ form is stable in native, hydrogenated, and transesterified beef and mutton fats and in their $Sat_3$ glycerides [5,163], but the $\beta'_L$ form, obtained by crystallisation from hexane in the presence of succinic acid, in hydrogenated cottonseed oil, where PalStPal predominates

(Table 6).[172] Transition to the stable polymorphic composition is accelerated by mixing fats rich in SatSatUnsat with fats containing much SatUnsatSat[150,168], and also by keeping at a constant temperature[154]. Thus several vegetable oils acquire a grainy texture during winter storage, owing to the direct transition of the $\alpha_L$ form of the $Sat_3$ glycerides into the $\beta_L$ form[4,170].

Study of the polymorphism of natural fats has made progress, and has shown in particular that their structure is not statistical. Further investigations should give a more complete picture of the polymorphic forms of triglycerides, especially the unsaturated compounds, which constitute the major part of the world production of fats, although their polymorphism has been little studied as yet[146]. Determination of the totality of properties of all crystalline modifications of corresponding synthetic and natural triglycerides is important for identification of the latter with respect to composition and structure[5]. The polymorphic properties of a fat are important also for its technical appraisal[164] with respect to consistency, plasticity, graininess, and other properties[142,143].

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