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Review

Synthesis and properties of glycerides

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The specific structure of glycerides can be of importance in the metabolism of lipids. Examples of this are seen in the specific distribution of fatty acids in animal and vegetable triglycerides (1), in the synthesis of triglycerides by liver and adipose tissue (2, 3), and in the deposition and mobilization of fatty acids in adipose tissue (4, 5). In this review we have assembled information on the chemistry of glycerides, which hopefully will assist those investigating the metabolism of these materials. This information is presented under four headings: synthesis, stability, isolation, and analysis.

The chemistry of glycerides is obviously too large a subject to be comprehensively treated in a single review. Consequently, we have emphasized those subjects that will be of use primarily to those interested in the metabolism of lipids. Moreover, in order to allow a critical discussion of the various techniques, the selection of subject matter has been influenced by the personal experience of the authors and their associates. Because of these considerations, many worth-while contributions are not included. These can be found in the more comprehensive review articles to which reference is made in each section.

I. Synthesis of Glycerides

A wide variety of methods for the synthesis of glycerides is discussed in the excellent review articles by Hartman (6) and by Malkin and Bevan (7). The first of these is particularly helpful because of the detailed presentation of the difficulties that can be encountered. In spite of this multitude of methods, no single, satisfactory procedure has been developed for the preparation of all types of glycerides. The following is a selection of those methods that we believe are the simplest with respect to equipment and manipulations and yet yield products of high purity. Most of these methods are adequately described in the cited literature references; where needed, additional details are supplied.

General Principles. A judicious choice of synthesis route is especially important in the preparation of partial glycerides to be used as intermediates in the synthesis of other gylcerides. In general it is advantageous to introduce the saturated fatty acid groups, if there are any, as early as possible in the synthesis. The presence of these acids permits purification by crystallization, which yields products of high purity with the minimum opportunity for isomerization.

The synthesis of a triacid[1] triglyceride of specific structure usually requires the successive synthesis of a monoglyceride, a diglyceride, and finally the triglyceride. Similarly the synthesis of diacid triglycerides and diglycerides normally proceeds through a monoglyceride intermediate; in a few instances this intermediate is not necessary. The preparation of monoacid diglycerides and triglycerides is accomplished directly without the use of intermediate partial glycerides.

The importance of using pure starting materials in the synthesis of glycerides cannot be overemphasized. Many of the aberrant analytical values reported for glycerides may well be the result of failing to assure adequate purity of the starting materials. This is a particular problem in the case of fatty acids, since many of the commercially available acids are far from pure. With the advent of gas-liquid chromatography,

[1] The terms triacid, diacid, and monoacid are used to designate glycerides containing three different fatty acids, two different fatty acids, and one kind of fatty acid, respectively.

however, it is now easy to detect the presence of contaminants. Glycerol of high purity is available commercially. Since it readily takes up water, it is usually necessary to dehydrate the glycerol before it can be used in the synthesis of glycerides. Distillation, in which the first and last 15% are rejected, is the most convenient method for accomplishing this end.

By use of the appropriate catalyst, free fatty acids, methyl esters, or fatty acid chlorides can be used to acylate glycerol or partial glycerides. Free fatty acids require an acid catalyst, such as p-toluenesulfonic acid or HCl. The reaction with methyl esters is an interesterification and a basic catalyst, such as sodium methoxide or NaOH, is used. A catalyst concentration of about 0.5% based on the weight of free fatty acid or methyl ester is optimal. Esterifications, starting with a fatty acid chloride, are carried out in the presence of pyridine (8).

For the preparation of up to 50 grams of product, the methods employing fatty acid chlorides are preferred. These have the advantage of: (*a*) simplicity, (*b*) uniformity of manipulations for the preparation of a wide variety of glycerides, (*c*) assuring that esterification takes place without isomerization (9), and (*d*) providing a high yield of product, an important consideration when the starting materials are in limited supply.

Preparation of Fatty Acid Chlorides. Thionyl chloride, oxalyl chloride, and phosphorous pentachloride can be used for the preparation of fatty acid chlorides. Of these, oxalyl chloride is preferred, since it can be used with unsaturated as well as saturated acids (10, 11), whereas the others can cause alterations in unsaturated acids. Youngs et al. (12), from a study of the preparation of fatty acid chlorides using phosphorous pentachloride, recommended water washing rather than distillation for purification. We have found water washing to be preferable to distillation regardless of the method used to prepare fatty acid chlorides.

The acid chlorides are prepared by mixing one part of the fatty acid with 1.2 parts of oxalyl chloride on a weight basis. The mixture is allowed to react for three days at room temperature, after which it is heated on a steam bath for 30 min under the vacuum of a water aspirator. Twenty volumes of hexane is added and the fatty acid chloride is washed three times with ice water, immediately dried with anhydrous sodium sulfate, and filtered. The solvent is removed by evaporation under vacuum. We have stored linoleoyl chloride at $-18°$ in an atmosphere of nitrogen for as long as two years without deterioration.

Acylation with Fatty Acid Chlorides. For successful acylation with fatty acid chlorides, the reaction must be carried out in a water-free system. Since esterification with acid chlorides will take place in the absence of alkaline or acidic catalysts and without heating (all of which promote isomerization), it is possible to esterify partial glycerides without acyl migration. The following is a generalized procedure for esterification with fatty acid chlorides.

Chloroform is washed with water to remove the alcohol it contains and is then dried with anhydrous sodium sulfate. Glycerol or the glycerol derivative is dissolved in an amount of purified chloroform sufficient to give a single phase. An amount of pyridine equal, on a molar basis, to the amount of fatty acid chloride to be used is added, followed by the acid chloride. The mixture is allowed to stand at room temperature for the time appropriate to the end product desired. The quantity of acid chloride and the reaction time are given subsequently for each glyceride type. The solution is then diluted with 20 vol of 1:1 petroleum ether–ethyl ether and washed as follows: twice with water, three times with 1% aqueous HCl, and three times with water. The organic phase is dried with anhydrous sodium sulfate and the solvent removed under vacuum.

SYNTHESIS OF MONOGLYCERIDES

The synthetic procedure for monoglycerides consists of esterifying glycerol that contains the appropriate blocking group, removing the blocking group, and then purifying the product. Since monoglycerides are readily isomerized by acid, alkali, or heat (usually to an 88 to 12 mixture of the 1- and 2-isomers, respectively), the conditions under which the synthesis is carried out must be carefully selected. Isomerization is most likely to occur after the blocking group has been removed; hence, the purification step requires particular care. Wherever possible, purification should be by crystallization, since this process affords the minimum opportunity for isomerization.

The esterification of a specific hydroxyl group of glycerol requires that the other two hydroxyl groups be blocked prior to the esterification. Several blocking groups have been proposed. Two of these are satisfactory for most preparations. When the acyl group is to be introduced into the primary position, 1,2-isopropylidene glycerol is used. If the fatty acid group is to be esterified with the secondary hydroxyl group, 1,3-benzylidene glycerol is used. The trityl (triphenylmethyl) group is another blocking agent that has been extensively employed, particularly by Verkade. An excellent summary of his work in this field is available (13). As will be discussed, trityl and benzyl ether have served as blocking groups in the preparation of optically active glycerides.

1,2-Isopropylidene glycerol is now available commercially. This reagent should be purified by distillation before use. Hartman (14) has recently published an improved method for the laboratory preparation of this material.

The following procedure for the preparation of 1,3-benzylidene glycerol is a modification of the method of Hibbert and Carter (15) and is similar to that used by Verkade (16). It has a product yield of 40–50%. This high yield removes the main limitation to the use of this compound in glyceride synthesis.

Equimolar amounts of dry glycerol and benzaldehyde, three volumes of toluene, and a catalytic amount of p-toluenesulfonic acid (2% of glycerol by weight) are stirred and heated at reflux temperature with the condensed vapors passing through a water trap. When water evolution has stopped, the flask is immersed in an ice water bath, allowed to cool, and the contents seeded with 1,3-benzylidene glycerol. Maximum yield is obtained if crystallization is allowed to proceed for at least 12 hr. The crystals are isolated by filtration and while still cold they are stirred with 8 vol of toluene containing sufficient sodium methoxide to neutralize the catalyst. The crystals are dissolved by warming; and the toluene solution is washed with 1% aqueous dibasic sodium phosphate, dried with anhydrous sodium sulfate, and crystallized at 0°. The isolated crystals are recrystallized from benzene–hexane 1:1. 1,3-Benzylidene glycerol has two melting points. Verkade and Van Roon (16) attribute the lower of these (63.5–64.5°) to the *cis* isomer and the higher (82.5–83.5°) to the *trans* isomer. We have found both isomers to be equally suitable for the synthesis of 2-monoglycerides. In recent years, however, we have been able to obtain only the lower melting form. Although 1,3-benzylidene glycerol is fairly stable, it should be recrystallized before use.

After acylation, removal of the blocking group from the glycerol must be accomplished without altering the fatty acid itself or causing it to migrate on the glycerol molecule. Fischer and Baer (17) used aqueous acetic or hydrochloric acid to remove the isopropylidene blocking group. This procedure probably causes some isomerization of the fatty acid to the 2-position. In view of the analytical values (17) obtained on monoglycerides prepared by this procedure, it appears that the contaminating isomer is removed by the subsequent crystallization.

The benzylidene group should not be removed by acid hydrolysis because isomerization in this instance will result in the unwanted 1-isomer being the major constituent. However, the blocking group can be removed by hydrogenolysis (18, 19, 20) without

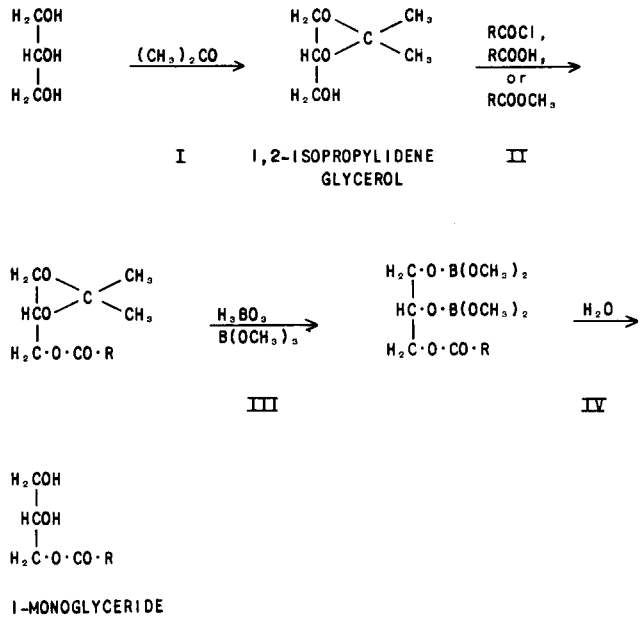

FIG. 1. Synthesis of 1-monoglyceride.

isomerization taking place. The method is not applicable, of course, to the preparation of unsaturated 2-monoglycerides because there would be conversion to the saturated counterpart.

The demonstration by Martin (21) that the benzylidene group can be replaced by diethyl borate has solved the problem of fatty acid alteration or isomerization of the partial glyceride. Removal of the diethyl borate poses no problem because it hydrolyzes from the glyceride on exposure to water at room temperature. Hartman (22) later extended this method to the removal of the isopropylidene blocking group. We have subsequently found trimethyl borate to be superior to triethyl borate for the removal of either blocking group. The benzylidene or isopropylidene group is replaced by dimethyl borate by heating a solution of the blocked monoglyceride in the presence of boric acid and trimethyl borate. The temperature is not allowed to rise above 100°. The reaction mixture is then washed with water to remove the dimethyl borate group.

1-Monoglycerides. In order that esterification of only one primary hydroxyl group will take place, 1-monoglycerides are prepared from 1,2-isopropylidene glycerol (Fig. 1). The acyl group may be introduced by way of the acid chloride, the free acid, or the methyl ester (Reaction II). Hartman (14) has described a procedure in which the preparation of isopropylidene glycerol (Reaction I) and its subsequent esterification are carried out stepwise in a single reaction chamber. Since an acid catalyst is used in the preparation of the

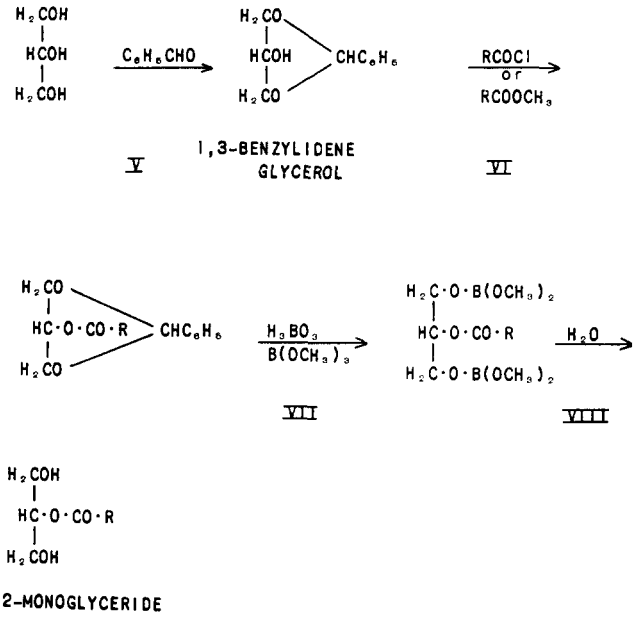

FIG. 2. Synthesis of 2-monoglyceride.

isopropylidene glycerol, free fatty acid must be used in the esterification reaction. Actually the same catalyst is used for both reactions. Hartman has questioned the use of this procedure with unsaturated fatty acids because of the discoloration that takes place during the esterification step. It has been our experience that some discoloration does take place; however, the colored contaminant is removed during purification and the final product has the proper analytical values. When isopropylidene glycerol is already available, 1-monoglycerides can be prepared by the same method, with the omission of Reaction I.

If a methyl ester is preferred as the starting material, a basic catalyst is used. The mixture of methyl ester, catalyst, and isopropylidene glycerol, and five volumes of solvent (usually toluene) is stirred under vacuum while a temperature of 80–100° is maintained. The rate of distillation of the liberated methanol and the toluene is controlled so that the reaction is continued for approximately 4 hours. Isopropylidene glycerol can also be esterified at room temperature in the presence of pyridine with a fatty acid chloride and a 24-hr reaction time. Maximum yield is obtained by all three routes when a 20% excess of isopropylidene glycerol is used. Regardless of the method of acylation, the blocking group is removed through the borate intermediate by the method of Martin (21) (Reactions III and IV).

Once the blocking group has been removed, the monoglyceride is readily isomerized by such agents as acid, alkali, or heat. This necessarily limits the methods suitable for purification. Most chromatographic procedures cause isomerization and consequently are of little value here. Of the methods available, crystallization is preferred. The monoglyceride is dissolved in 40–60 volumes of petroleum ether and crystallized at the appropriate temperature; e.g., monolinolein at −35°, monoolein at −25°, and saturated monoglycerides at 0°. The product is collected by filtration and traces of solvent are removed under vacuum without heating. The storage temperature should be 0° or less.

2-Monoglycerides. 2-Monoglycerides are prepared by first blocking the primary hydroxyl groups with benzaldehyde (Fig. 2, Reaction V). The earliest preparation of saturated 2-monoglycerides was from 1,3-benzylidene glycerol and fatty acid chloride (18, 19, 20) (Reaction VI). Since preparation of fatty acid chlorides is somewhat laborious, it is simpler to use the methyl ester of the fatty acid and an alkaline catalyst to obtain saturated monoglycerides. As in the preparation of 1-monoglycerides, the reaction is carried out at 80–100° under a vacuum for 4 hr. Although the blocking group of the final product can be removed by hydrogenolysis, the borate replacement method of Martin (21) is far simpler (Reactions VII and VIII).

For the preparation of unsaturated 2-monoglycerides, it is best to acylate the blocked glycerol with the acid chloride. The details of a satisfactory method have been published by Martin (21). A 24-hr reaction time at room temperature using a 20% excess of 1,3-benzylidene glycerol gives a high yield of product. The only known means by which the blocking group can be removed without altering the fatty acid or isomerizing the 2-monoglyceride is by borate replacement (21).

As in the case of 1-monoglycerides, it is best to purify the 2-monoglycerides by crystallization. The same precaution in storing and handling should be observed as with 1-monoglycerides.

SYNTHESIS OF DIGLYCERIDES

Satisfactory methods for synthesizing many of the diglycerides have been slow to develop owing to the lack of a suitable quantitative method for characterizing the products. The recent application of hydrolysis with pancreatic lipase to this problem is the first satisfactory method (9, 23). Differential thermal analysis in association with fractional crystallization (24) and thin-layer chromatography (25) can be used to obtain semi-quantitative values.

With the exception of certain monoacid 1,3-diglycerides, a monoglyceride of known structure is the preferred starting material in the synthesis of diglyc-

erides. Because of the readiness with which monoglycerides isomerize, esterification is limited to the use of fatty acid chlorides. Moreover, Crossley et al. (24) recently demonstrated that diglycerides also isomerize readily, with the equilibrium mixture being approximately a 60-40 mixture of 1,3- and 1,2-diglycerides. Thus the conditions of esterification and purification used in the preparation of diglycerides must be such as to minimize the opportunity for isomerization of the product. In many synthetic methods, the starting monoglyceride and the resulting diglyceride are exposed to acid, alkali, or heat. The ease with which mono- and diglycerides isomerize under the influence of these agents suggests that it would be desirable to redetermine the constants that have been reported for many of these supposedly pure diglycerides (24).

1,2-Diglycerides. Disaturated, monoacid diglycerides have been prepared by Howe and Malkin (26) using a modification of the method originally reported by Sowden and Fischer (27). In this procedure, the free hydroxyl group of 1,2-isopropylidene glycerol is blocked with benzyl ether. The isopropylidene is removed and the free hydroxyl groups (1- and 2-positions) are acylated with a fatty acid chloride. Since two hydroxyl groups are being esterified simultaneously, this method is limited to the preparation of monoacid diglycerides. The benzyl group is removed by hydrogenolysis, which limits the method to the preparation of disaturated diglycerides.

We have found that incomplete acylation of 2-monoglyceride followed by chromatographic separation of the 1,2-diglyceride from the resulting mixture of glycerides is an efficient procedure for the preparation of diunsaturated, disaturated, and mixed saturated-unsaturated 1,2-diglycerides. Although not as elegant as many other methods, it presents a single method by which 1,2-diglycerides with a wide range of fatty acid compositions can be prepared. The appropriate 2-monoglyceride is acylated as described earlier at room temperature by adding an equimolar amount of the fatty acid chloride. The reaction is allowed to proceed for only 4 hours. The final reaction mixture consists of free fatty acid, 2-monoglyceride, 1,2-diglyceride, and triglyceride; however, under these conditions the diglyceride constitutes at least 50% of the mixture. The diglycerides are isolated by silica gel chromatography (28). The small amount of contaminating free fatty acid is removed by dissolving the diglycerides in wet ethyl ether and adding, with stirring, the ion exchange resin Amberlite 1RA-400 (OH) (29). Although this method of synthesis is somewhat wasteful of starting materials, it is the only one by which 1,2-diglycerides, in which one or both of the fatty acids are unsaturated, can be prepared. It has the advantage of simplicity of manipulation. In Tables 1 and 3 the 2-monoglycerides that would be used in preparing a number of 1,2-diglycerides are shown.

TABLE 1. SYNTHESIS OF DIGLYCERIDES

Reaction*	Product
1,2-Isomer†	
–P– + SCl	SP–
–S– + SCl	SS–
–O– + OCl	OO–
–L– + OCl	OL–
1,3-Isomer‡	
S– – + PCl	S–P
S– – + SCl	S–S§
O– – + OCl	O–O
O– – + LCl	O–L

* S = stearic, P = palmitic, O = oleic, L = linoleic, – = free OH group. Abbreviated descriptions of glycerides represent the structure; thus, SP– is 1-stearoyl 2-palmitin.

† Reactions yield a mixture of glycerides. Product is isolated by chromatography.

‡ Reactions yield a mixture of glycerides. Product is isolated by chromatography and crystallization.

§ Can also be prepared by directed interesterification.

1,3-Diglycerides. The method used for the preparation of a 1,3-diglyceride depends on the types of fatty acids that are to be in the product. There is need for a simpler and more straightforward method for synthesizing this class of glycerides. The conditions of esterification result, in all instances, in a mixture of mono-, di-, and triglycerides with both 1,3- and 1,2-diglycerides being present. The higher melting 1,3-diglycerides can be obtained by directed interesterification. In the case of those having a low melting point, both diglycerides are isolated by silica gel chromatography (28). The 1,3-isomer is readily obtained by crystallization, since it has one-tenth the solubility of the 1,2-isomer (24). The following methods, although far from ideal, will result in products of high purity, in fair yield.

Monoacid 1,3-diglyceride, whose component fatty acid has a melting point in excess of 20°, is most easily prepared by directed rearrangement as described by Baur and Lange (30). The pure monoacid triglyceride of the desired fatty acid, triacetin, and glycerol are mixed in the molar ratio of 0.5:1.0:0.8. A basic catalyst is added and the mixture heated above its melting point for 2 hr. The temperature is then decreased slowly so that the preferential crystallization of the 1,3-diglyceride of the high molecular weight fatty acid results in directed interesterification to this product. The catalyst is inactivated with acetic acid and the 1,3-diglycerides recovered by filtration and purified by crystallization. Using this procedure, we

have obtained 1,3-dipalmitin and 1,3-distearin of better than 98% purity.

1,3-Diglycerides containing two different saturated acids or a saturated and an unsaturated acid can be prepared by the acylation of the appropriate 1-monoglyceride with the appropriate fatty acid chloride. The conditions for incomplete acylation with a fatty acid chloride are the same as those described for the preparation of a 1,2-diglyceride. The reaction products are a mixture of 1-monoglyceride, 1,2- and 1,3-diglycerides, and triglycerides. The diglycerides are separated from the other glycerides by silica gel chromatography (28). The 1,3-diglycerides are readily separated from the 1,2-diglycerides by crystallization from ten volumes of hexane at the appropriate temperature; 35° for distearin and dipalmitin, −20° for diolein (24). The filtrate from this crystallization is predominantly 1,2-diglyceride with a small amount of 1,3-diglyceride. The over-all yield of 1,3-diglyceride can be increased by removing the solvent from the filtrate and holding the residue at a temperature of 165° for 1 hour. The heating isomerizes the diglycerides to a 60/40 mixture of the 1,3- and 1,2-diglycerides. The 1,3-diglycerides are isolated by crystallization as before.

By far the most difficult glyceride to synthesize is the 1,3-diglyceride that contains two unsaturated fatty acids. In the syntheses described above, the diglycerides contain at least one saturated fatty acid. Consequently the character of the crystals is such that the 1,3-diglyceride can be readily separated from the 1,2-diglycerides. Where both fatty acids are unsaturated, much of this distinguishing character is lost and purification by crystallization is difficult. We have, however, successfully prepared 1,3-diolein by crystallizing it from the mixture of dioleins using ten volumes of hexane and a temperature of −20°. On the other hand, attempts to prepare 1,3-dilinolein by this method have not been successful. In Table 1 are listed the preferred monoglycerides for the synthesis of some 1,3-diglycerides.

SYNTHESIS OF TRIGLYCERIDES

Monoacid triglycerides are relatively simple to synthesize since isomerization of the glycerides is not a problem. Although other starting materials will serve, fatty acid chlorides have certain advantages that recommend them for the preparation of small amounts of material. A molar ratio of one glycerol to four fatty acid chloride in pyridine and chloroform is refluxed for 4 hours. Purification follows the general procedure of washing and crystallization that has already been described.

Mixed acid triglycerides are prepared from the appropriate partial glyceride intermediate. Since heat or the usual acidic or basic catalysts causes isomerization of the partial glyceride intermediates, fatty acid chlorides are used for acylation. Suggested synthetic routes to various diacid triglycerides are given in Table 2. By using the partial glycerides listed in this table, only a single acylation step is required. A 50% molar excess of the fatty acid chloride that would be required for complete acylation is used and the reaction is allowed to continue at room temperature for 72 hr. These conditions will result in complete acylation with no isomerization (9).

TABLE 2. SYNTHESIS OF DIACID TRIGLYCERIDES

Reaction*	Product
–S– + OCl	OSO
S–– + OCl	SOO
–L– + OCl	OLO
L–– + OCl	LOO
S–S + OCl	
or	
–O– + SCl	SOS
O–– + SCl	OSS

* S = stearic, P = palmitic, O = oleic, L = linoleic, – = free OH group. Abbreviated descriptions of glycerides represent the structure; thus, OSO is 2-stearoyl diolein.

The preparation of a triacid triglyceride requires the successive synthesis of a monoglyceride, a diglyceride, and finally the triglyceride. The series of reactions used in the preparation of a number of triacid triglycerides is given in Table 3. Here too, complete acylation of the diglyceride is achieved by using a 50% molar excess of the fatty acid chloride and a reaction time of 72 hr at room temperature. The preparation of a triglyceride in which all three fatty acids are unsaturated is the most difficult because of the problems

TABLE 3. SYNTHESIS OF TRIACID TRIGLYCERIDES

First Reaction*,†	Second Reaction	End Product
–S– + PCl → PS–	PS– + OCl →	PSO
–O– + SCl → SO–	SO– + PCl →	SOP
–P– + SCl → SP–	SP– + OCl →	SPO
–S– + OCl → OS–	OS– + LCl →	OSL
–O– + SCl → SO–	SO– + LCl →	SOL
–L– + SCl → SL–	SL– + OCl →	SLO

* The first reaction yields a mixture of glycerides. The 1,2-diglyceride component is isolated by chromatography.

† S = stearic, P = palmitic, O = oleic, L = linoleic, – = free OH group. Abbreviated descriptions of glycerides represent the structure; thus, PSO is 1-palmitoyl 2-stearoyl 3-olein.

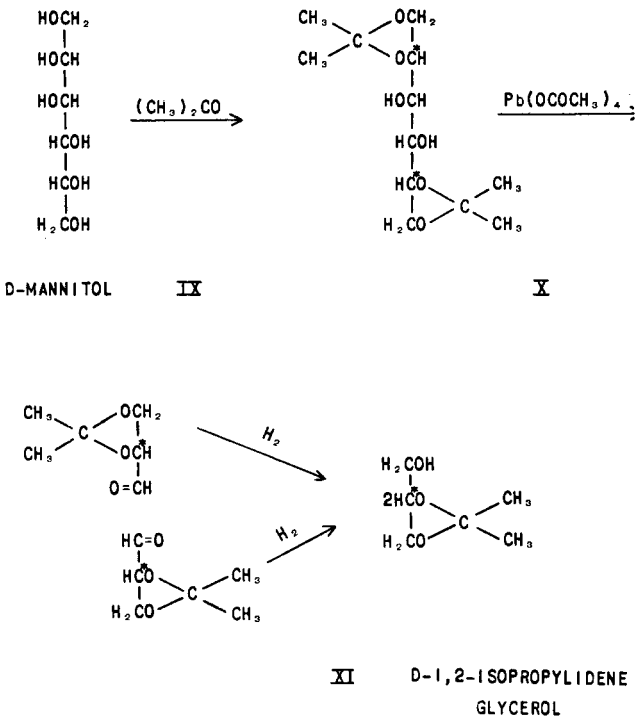

FIG. 3. Synthesis of D-isopropylidene glycerol.

in synthesizing the appropriate mono- and diglyceride intermediates.

SYNTHESIS OF OPTICALLY ACTIVE GLYCERIDES

The synthesis of D- (31, 32) and L- (33) isopropylidene glycerol by Baer and Fischer made possible the preparation of optically active partial glycerides. The D- and L-isopropylidene glycerols are obtained from D- and L-mannitol by methods that transfer, without loss, the asymmetry of carbon atoms 2 and 5 of the mannitols (Fig. 3). The preparation of these intermediates and of optically active mono- and diglycerides has been the subject of a review by these authors (34). Asymmetric triglycerides do not have a measurable optical activity (32).

Optically active monoglycerides are prepared by acylation of the isopropylidene glycerol with a fatty acid chloride (Fig. 4, Reaction XII) followed by removal of the isopropylidene group by the action of hydrochloric acid in the cold (35) (Reaction XIII). The analytical values obtained on these monoglycerides show them to be the 1-isomer. Yet one might expect the presence of approximately 12% of 2-monoglyceride due to isomerization by the hydrochloric acid. Baer

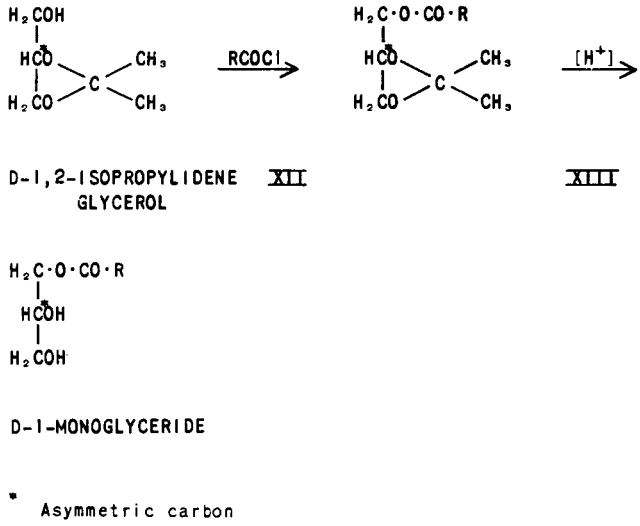

FIG. 4. Synthesis of optically active monoglycerides.

and Fischer (35) offer certain evidence to support their position that the acid treatment does not cause isomerization. The possibility remains that the 2-isomer is formed, but, in the process of purifying the monoglycerides, only the 1-isomer, which is the optically active species, is isolated.

For the synthesis of optically active diglycerides, the D- or L-isopropylidene glycerol is used to prepare 1-benzyl glycerol ether (26) (Fig. 5, Reactions XIV and XV). Saturated, monoacid diglycerides are obtained by acylation with fatty acid chloride (Reaction XVI) and removal of the benzyl group by hydrogenolysis (36, 37) (Reaction XXI). It is the last step that limits this method to the preparation of saturated diglycerides.

Baer and Buchnea (38) have followed the same reaction sequence in the preparation of D-1,2-diolein and L-1,2-diolein. To protect the double bond of the oleic acid during the hydrogenolysis step, the oleic acid is first brominated. After removal of the benzyl group, the oleate is debrominated with activated zinc, which is reported to result in a minimum amount of *cis-trans* isomerization.

This approach to the preparation of optically active diglycerides has now been extended by Buchnea and Baer (39) to the synthesis of diacid diglycerides. The fatty acids are introduced in a four-step process starting with optically active 1-benzyl glycerol ether. Acylation is carried out with a fatty acid chloride. The successive steps are: (*a*) tritylation of the 1-benzyl glycerol ether (Fig. 5, Reaction XVII), (*b*) acylation of the remaining free hydroxyl group, which is in the 2-position (Reaction XVIII), (*c*) removal of the trityl group with a simultaneous shift of the fatty acid from the 2- to the 1-position by hydrogen chloride in petro-

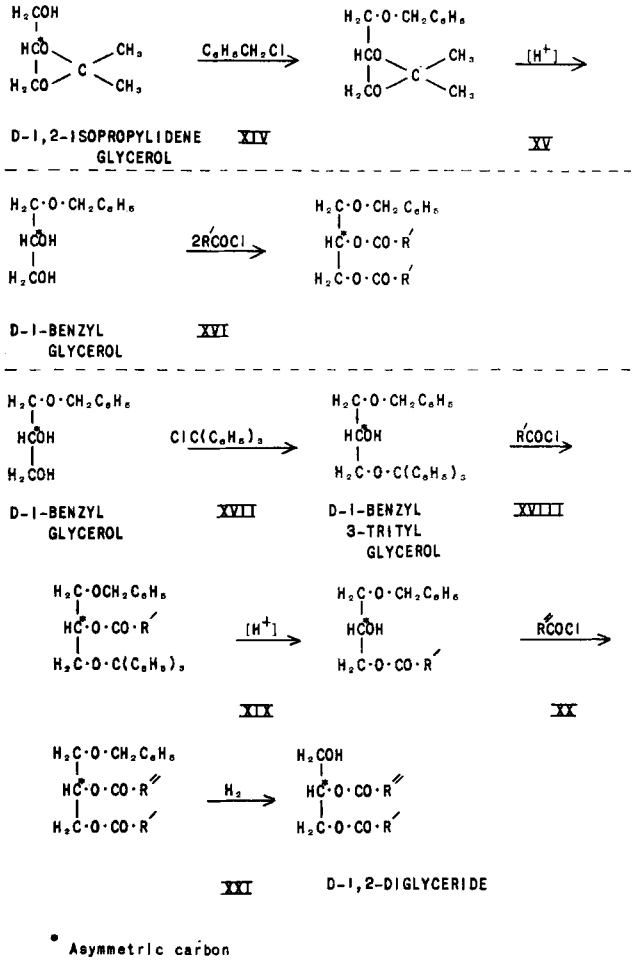

FIG. 5. Synthesis of D-1,2-diglyceride.

leum ether (Reaction XIX), and (*d*) introduction of the second fatty acid into the 2-position (Reaction XX). Finally, the benzyl group is removed by hydrogenolysis (Reaction XXI). If an unsaturated fatty acid is used in the synthesis, its double bonds must be protected during hydrogenolysis by first brominating the fatty acid. The last step then is the removal of the bromine with zinc.

In this synthesis, too, there is the problem of the presence of two isomers after the removal of the trityl group with acid (Reaction XIX). The results obtained by Buchnea and Baer led them to conclude that there is complete isomerization of the fatty acid to the 1-position. However, it seems likely that about 12% of the 2-isomer would remain. Here again, as was discussed above in the case of the preparation of optically active monoglycerides, the purification step may remove the unwanted 2-isomer. The authors report a yield of only 50% in this reaction; a portion of the missing material could be the 2-isomer. Thus far no one has attempted the synthesis of optically active glycerides using the methods developed by Baer and his colleagues in conjunction with the borate replacement method of Martin (21). This combination appears to offer a simpler approach to the preparation of some of these compounds.

II. STABILITY

Glycerides containing an unsaturated fatty acid must be protected from oxidation by excluding such agents as metals, oxygen, heat, and light. Consequently they are best stored under nitrogen in tightly sealed containers in the cold. Stability can be assured further by the addition, at a level of about 50 parts per million, of antioxidants, such as butylated hydroxyanisol, butylated hydroxytoluene, or nordihydroguaiaretic acid. Another, and probably the most effective, is hydroquinone. It has the advantage that it can be removed from the glycerides by water-washing. However, in contrast to the other antioxidants mentioned, hydroquinone is not approved for human consumption.

The partial glycerides pose a special problem because of the ease with which they isomerize. The section on synthesis has pointed out the problems that exist in the preparation of glycerides as the result of this instability. This also has its counterpart in the biological area where isomeric form is usually of importance. Although some information has been obtained, our knowledge about the isomerization of partial glycerides and the conditions that bring it about is limited.

Monoglycerides. The studies of Daubert and King (40) and Stimmel and King (19) led to the conclusion that when a 2-monoglyceride is exposed to acid, alkali, or heat, there is complete conversion to the 1-isomer. However, Verkade and van Lohuizen (41, 42) subsequently found that under acidic conditions, monoglycerides of aromatic acids exist in equilibrium with a composition of 88% 1-monoglyceride and 12% 2-monoglyceride. Martin (43) demonstrated that this same ratio exists for monoglycerides of long-chain fatty acids. Moreover, his demonstration that this equilibrium mixture can be readily attained by treating monoglycerides with perchloric acid formed the basis of the method for determining the total monoglyceride and 2-monoglyceride content of a fat. Heating, and a variety of other treatments, of 1- or 2-monoglycerides results in isomerization (44) and the equilibrium attained (45) is the same as that reported by Martin. Monoglycerides should be stored at 0°, or less, since isomerization takes place slowly even in the absence of a catalyst (46, 47). Because of the rapidity with which

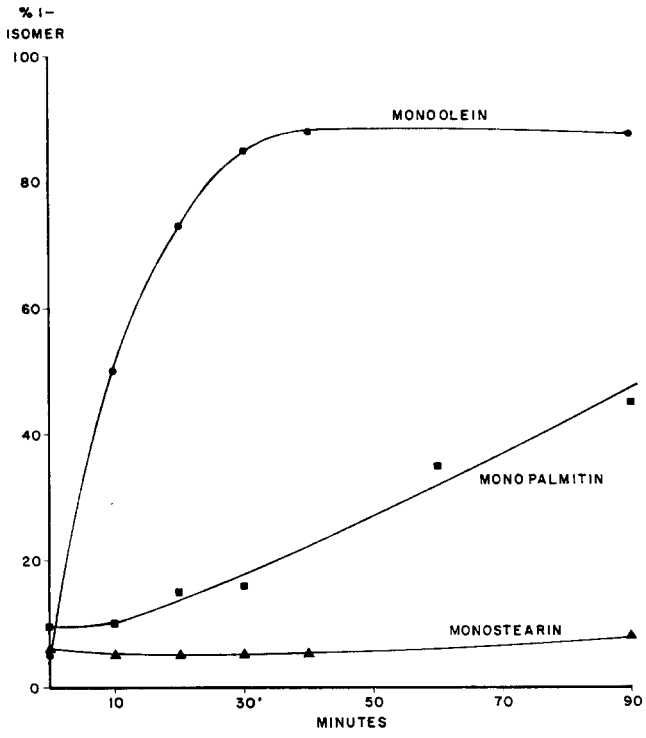

FIG. 6. Isomerization of 2-monoglycerides at 40° in 1.0 M Tris buffer, pH 8.0.

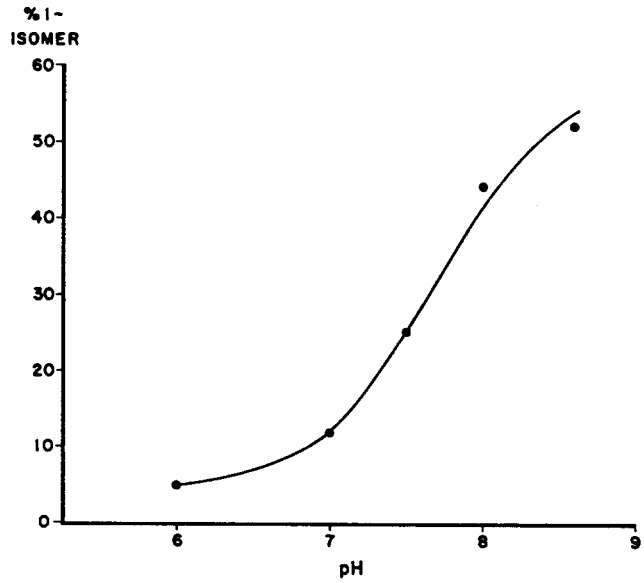

FIG. 7. Isomerization of 2-monoolein in lysine–histidine buffer (pH 6 to 8) or Tris buffer (pH 8 and 8.6) at 26°, 30 minutes exposure.

they are isomerized by heat, stored material should not be melted for the purpose of sampling.

In many of the synthetic methods, acid is used either to remove blocking groups or to convert the 2-isomer to the 1-isomer. This is done on the principle that such treatment results in only 1-monoglyceride being present. Since it has been estabilished that 12% of 2-monoglyceride is present at equilibrium, the 1-monoglyceride obtained by these methods may contain appreciable amounts of the 2-isomer. On the other hand, the early recognition that acid causes isomerization of 2-monoglycerides led to the rejection of this reagent as a means for removing blocking groups in the preparation of this isomer.

In recent years, silicic acid chromatography has been widely used for the fractionation of lipids. It has been demonstrated that a variable portion of the 2-monoglyceride that is passed through such columns is isomerized to 1-monoglyceride (48, 49). It is reasonable to assume that 1-monoglyceride is converted to 2-monoglyceride under the same conditions. We have observed isomerization of monoglycerides on Florisil columns.

Molecular distillation can lead to isomerization as shown by Privett et al. (50), who found the second distillate of pure 1-monopalmitin to contain approximately 12% 2-monopalmitin. In contrast with the results obtained on silicic acid columns, this same paper contains evidence that monoglycerides do not isomerize on silica gel thin-layer chromatographic plates.

All of the above investigations have been carried out on the fat itself or the fat dissolved in an organic solvent. However, most biochemical reactions occur in an aqueous medium. To determine the effect of such an environment on isomerization, we added small amounts of 2-monoglycerides to 1.0 M, pH 8.0, tris (hydroxymethyl) aminomethane buffer and shook the suspension for 1 hour at 40°. The percentage of 1-isomer present after various time intervals is shown in Figure 6. In 40 min, the 2-monoolein was isomerized to the equilibrium mixture for monoglycerides. The slower rate at which the 2-monopalmitin isomerized, and the marked stability of the 2-monostearin, may be related to the melting point of these fats, which would influence their dispersibility in the aqueous phase.

The rate of isomerization varies with the pH of the system. Figure 7 shows the amount of 1-monoolein found after shaking 2-monoolein for 30 min in buffers of differing pH values at 26°. Although the rate of isomerization was found to be slowest at pH 6.0, the use of more acidic solutions undoubtedly would result in higher rates of isomerization, in keeping with the use of acid catalysts for isomerization. The temperature dependence of the reaction can be seen by comparing Figures 6 and 7. At the same pH of 8.0 and 30 min incubation, 85% of the monoolein was isomerized at 40°; whereas at 26°, only 44% conversion took place. Since the above conditions do not vary

greatly from those that are frequently used in biochemical studies *in vitro*, it is apparent that the possibility of isomerization must be considered in such experiments.

Diglycerides. Although diglycerides isomerize under many of the same conditions as do monoglycerides, they apparently are somewhat more stable. The presence of an equilibrium mixture of 1,2- and 1,3-diglycerides was suggested many years ago (51). Only recently, due to the development of new analytical methods, has it been possible to determine the ratio of the 2-isomers. Using a combination of differential thermal analysis and crystallization, Crossley et al. (24) have shown that the equilibrium mixture consists of 58% 1,3- and 42% 1,2-diglyceride. We have confirmed these values using the pancreatic lipase technique (9).

As in the case of the monoglycerides, heat, acid, or alkali cause acyl migration in diglycerides (24). Similarly, molecular distillation also causes isomerization (50). We have observed a rapid isomerization of diglycerides on Florisil columns. On the other hand, 1,2-diglycerides do not isomerize when they are passed through a silicic acid column (36, 38, 49).

We have found diglycerides to be more stable in aqueous dispersions than are monoglycerides. For example, shaking 1,2- or 1,3-diolein with buffer solutions at 40° for 30 min over a pH range of 6–8 caused little, if any, isomerization. In spite of this greater stability of the diglycerides, it still is necessary to establish that other systems will not bring about isomerization. For example, no information is available as to the effect various inorganic ions may have on isomerization. Until such information is forthcoming, it will be necessary to establish that any manipulation to which partial glycerides are subjected does not result in acyl migration.

III. ISOLATION

The methods for isolating and analyzing glycerides are not very satisfactory. At the present time it is possible to resolve mixtures of mono-, di-, and triglycerides and separate the two diglyceride isomers. However, no one has succeeded in separating 1-monoglycerides from 2-monoglycerides by means of chromatography. Only very simple glyceride mixtures can be fractionated on the basis of their fatty acid composition. The need for a method that would adequately accomplish this kind of separation is obvious. The fractionations that have been obtained show that glycerides follow the same pattern as do free fatty acids and methyl esters (52). Thus the introduction of one double bond into one of the fatty acids of a glyceride alters its separation characteristics, in many of the methods, to the same extent as does shortening, by two carbons, the chain length of one of the fatty acids.

In 1961, the American Oil Chemists' Society sponsored a series of lectures on fat analysis that has been published (53). A wide range of analytical and isolation techniques is covered in these papers. The authors have each had extensive experience with the methods they discuss and as a consequence offer a wealth of detailed information.

Crystallization. Low-temperature solvent crystallization was among the early methods used for the separation and fractionation of glycerides. The method is tedious and the yields may be poor. However, there are many areas of glyceride chemistry where it is an invaluable tool. Its prime advantage is that the manipulations involved are the ones least likely to cause alteration of the lipids. Much of the early work on the structure of glycerides in natural fats relied on this method (54, 55). One of the most valuable applications of crystallization at the present time is in connection with the synthesis of glycerides. Numerous instances of the use of this technique are given above in the section on synthesis.

Fractionation by crystallization can, however, be a source of error. For example, the classical method of removing phospholipids from lipid mixtures by precipitation from acetone in the cold will also result in the precipitation of any trisaturated triglycerides that are present in the sample. Similarly 1,3-diglycerides precipitate from hexane at a higher temperature than do 1,2-diglycerides (24). Consequently, when samples are exposed to temperatures that allow crystallization, one must ascertain whether a selective crystallization has led to an inadvertent fractionation.

Liquid-Liquid Extraction. Countercurrent distribution should be a good method for fractionating lipids because the separation is carried out in an inert atmosphere without the application of heat. Hence, there is minimum opportunity for alterations of glycerides during isolation. However, its usefulness is limited to the separation of glycerides that have rather marked differences in polarity. Recently the method has been described in detail by Scholfield (56).

Where differences in polarity exist, as in mono-, di-, and triglycerides, separation is relatively simple. Monoglycerides can be isolated from a mixture of glycerides by a four-stage completion of squares using 80% ethanol and Skellysolve F. The diglycerides are then obtained by a 12-stage distribution using 95% methanol and isooctane (57).

Dutton and his collaborators (58) have attempted to separate by liquid-liquid extraction the various glycerides that are found in natural fats. The shortcom-

ings of this method are illustrated in the report describing the fractionation of cocoa butter (59). In spite of the fact that the glyceride composition of this fat is relatively simple, the separation was only partly successful.

Countercurrent distribution probably has its greatest applicability when relatively large quantities of mono-, di-, and triglycerides are to be separated from each other. When smaller quantities are being handled, other methods that are more suitable because of speed and simplicity of the equipment are available.

Gas-Liquid Chromatography. Thus far, gas-liquid chromatography has not proved to be of particular value in the analysis of glycerides. A recent review by Huebner (60), although suggesting possible applications, does not offer a great deal of encouragement on the basis of reported successes. Simple mixtures of monoacid triglycerides will separate on suitable columns; however, natural triglycerides are made up of such a wide variety of triglycerides that these are only partially resolved (60, 61).

Some success has been obtained in applying gas-liquid chromatography to the measurement of partial glycerides. This is best accomplished with a derivative that increases the volatility of the partial glyceride. Huebner (62), for example, used the acetylated products of mono- and diglycerides. Another method is to convert monoglycerides to their allyl esters (63). Horrocks and Cornwell (64) have developed a method that allows the simultaneous determination of glycerol content and fatty acid composition. In this, glycerides are treated with lithium aluminum hydride, the resulting lithium aluminum alcoholates are acetylated, and these are analyzed by gas-liquid chromatography. Although the method does not yield any information about the structure of the glycerides, its greatest use may be for determining glycerol content and glycerol to fatty acid ratios.

Paper and Glass Paper Chromatography. The initial introduction of reverse phase chromatography of lipids by Kaufmann and Nitsch (65) was followed shortly by the use of paper which had been impregnated with silicic acid (66, 67). A detailed description of this procedure and its applications has appeared recently (68). As with most chromatographic procedures, excellent resolution of mono-, di-, and triglycerides is obtained. Separation of simple triglyceride mixtures can be obtained (69), although it seems improbable that the resolving power of this technique is sufficiently great to allow the fractionation of the glycerides of natural fats.

At about the time that separation of lipids by paper chromatography was being demonstrated, the same resolution was shown to occur on glass paper. By this technique, lipid classes can be separated (70) as can mono-, di-, and triglycerides (71). The details of this technique were reviewed recently (72).

Neither paper nor glass paper has been demonstrated to have any particular advantage. On the other hand, certain techniques, such as thin-layer chromatography, appear to be superior to these.

Thin-Layer Chromatography. Even though thin-layer chromatography is among the more recent methods to be introduced into the field of lipid chemistry, it has already shown itself to be of great value. Although the technique itself had been known for a number of years, and has found extensive application in the field of terpenes (73), it was not until 1956 through the work of Stahl (74) that its use in the lipid field was established. Since that time, its use has spread rapidly and numerous publications reporting studies on this technique have appeared. Mangold (75), in a review article describing this technique, gives detailed description of the equipment and the manipulations and examples of applications. By this method, it is possible to separate a wide range of lipid types. Although small samples are usually employed, multiple applications to a single plate afford total sample loads of up to 50 mg. The equipment and manipulations are relatively simple. Moreover, the chromatograms can be developed quite rapidly, usually in 5–30 minutes.

Mono-, di-, and triglycerides are readily separated by thin-layer chromatography (25, 50, 76). A ready separation of 1,2- from 1,3-diglycerides can also be obtained (25). The authors state that the latter separation is accomplished without isomerization. On the other hand, as is the case with other separation techniques, 1-monoglycerides cannot be separated from 2-monoglycerides.

Attempts to resolve by thin-layer chromatography the mixture of triglycerides present in natural fats have not been too successful. Relatively simple mixtures of triglycerides having a considerable difference in fatty acid composition can be separated (77). However, when the same technique was applied to corn oil, only eight spots were obtained on the chromatogram. Since it is likely that corn oil contains more than 20 different triglycerides, it is obvious that resolution was into classes of triglycerides and not into individual species of triglycerides.

The high sensitivity of thin-layer chromatography, together with the short time that the procedure takes, makes it an ideal method for checking the purity of lipid preparations (50). Figure 8 illustrates this. Chemical analyses showed the triolein designated "Before" to be contaminated with monoglyceride and

free fatty acid. Based on these analyses, the triolein was calculated to be 98% pure. However, the chromatogram revealed the presence of diglycerides, methyl ester, and an anti-oxidant as well. Since the nature of the contaminants was known, it was possible to purify the triolein so that it yielded the chromatogram designated "After"; this triolein is estimated to be "99.44% pure." In this instance, thin-layer chromatography not only revealed the presence of unknown impurities, but the detectable contaminants

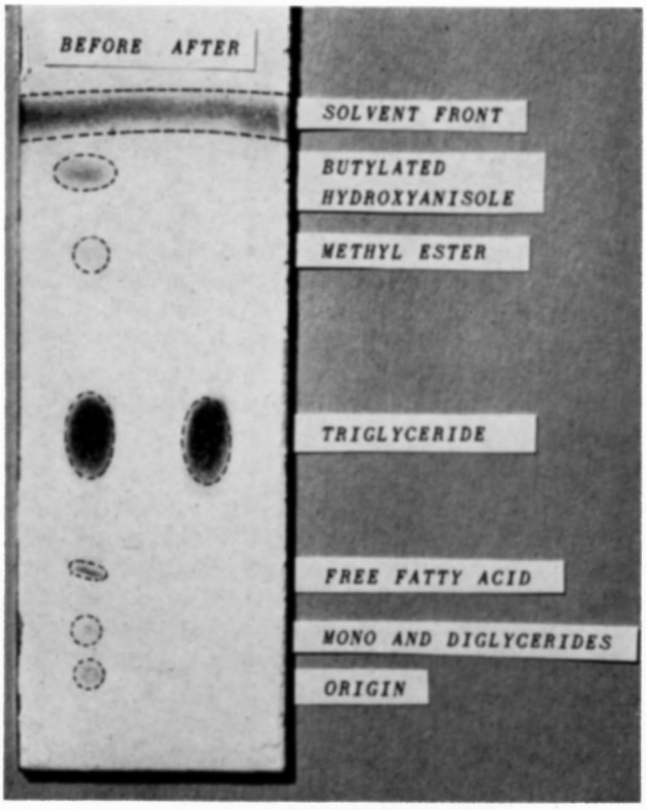

FIG. 8. **Thin-layer** chromatograms of synthetic triolein before and after purification.

were recognized in far less time than that required for the chemical analyses.

Several methods have been suggested for the quantitative determination of lipid components separated by thin-layer chromatography or by paper chromatography. The analysis can be carried out either on a fraction that has been removed from the chromatogram or by direct determination on the chromatogram itself. When an isolated fraction is analyzed, the total ester groups can be determined by reaction with hydroxamic acid (76), or total glycerol can be determined by the method of Lambert and Neish (78) after hydrolysis. Direct determination on the chromatogram is accomplished by measuring the density of the spot after rendering it visible, e.g., by charring (25). These methods suffer from the usual shortcoming of having high error values when chromatography is used for quantitative purposes. When the material being analyzed consists of a relatively simple mixture of known components, fairly accurate values are obtained; the more the unknown sample differs from this ideal, the greater is the error. As a consequence, the overall accuracy is about 85–90% with the experimentally determined value usually being lower than the true value.

Column Chromatography. Column chromatography is the method most widely used for the fractionation of lipids. Initially many of the adsorbents that were taken from nonlipid fields were found to be of little value. For example, alumina cannot be used because it causes isomerization of double bonds, hydrolysis of ester linkages, and isomerization of partial glycerides. With the introduction of silicic acid columns (79, 80), the separation of various lipid classes was readily accomplished. This adsorbent is the one most widely used, although subsequently others have been introduced that have advantages for certain systems.

Thus far, it has not been possible to rigorously standardize column chromatographic methods so that techniques used in one laboratory can be transferred without change to another laboratory. As a consequence, each laboratory has developed procedures particularly suited to its own needs. This situation has resulted in the reporting of numerous variations in chromatographic techniques. In spite of the multitude of reports, it remains necessary for each investigator to standardize his technique to his own particular circumstances. For the proper testing of column performance, pure lipid standards are needed. The sources of materials of suitable purity for this purpose are limited. Consequently many laboratories have found it best to prepare their own standards.

The selection of the proper eluting solvents can be made only by empirical testing. The elutropic ranking of solvents is of help in choosing the general types of solvents that might be used (81, 82, 83). Another technique useful in evaluating solvents is a spot test that requires only a small amount of material and a few minutes (84, 85). Warner and Lands (86) have developed a nomogram for solvents used in gradient elution systems. A paper by Rouser, et al. (87), although dealing mainly with the fractionation of phospholipids, lists a number of precautions and manipulative techniques that are applicable to all types of lipids.

Following the introduction of silicic acid column chromatography in the lipid field (82, 88), Borgström (89) demonstrated the separation of lipid classes on such columns with petroleum ether and benzene. Another solvent system has been recommended by Fillerup and Mead (90). Borgström (49) also established that mono-, di-, and triglycerides can be separated from each other on silicic acid columns. The range of materials that can be fractionated by this technique and numerous refinements in the procedure were subsequently described by Hirsch and Ahrens (48). An excellent review of these and subsequent papers has been published by Wren (91). His paper also contains an extensive discussion of the theory and use of silicic acid chromatography.

The fractionation of triglycerides has been attempted on such columns. The separation obtained in model systems containing triglycerides of very different fatty acid compositions was good; but when the separation was attempted with natural fats, insufficient resolution was obtained (92). As discussed in the section above on stability, monoglycerides partially isomerize on silicic acid columns while diglycerides apparently are not affected.

Carroll (93) has recently reported the use of Florisil columns for the separation of lipids. This adsorbent allows a more rapid separation of glycerides than can be made on silicic acid. Moreover, fatty acids are eluted after the glycerides, rather than simultaneously with the tri- and diglycerides as is the case with silicic acid. This is a promising new technique, but it has not been sufficiently evaluated for the alterations it may induce in lipids. We have observed that it causes isomerization of both mono- and diglycerides.

A number of nonpolar compounds have been investigated by Hirsch (94) as possible stationary phases for column chromatography. Three of these, gum rubber, polystyrene-divinylbenzene, and polymerized vegetable oil, were the most useful. Besides the usual application to lipid classes, the separation of the triglycerides of linseed oil was attempted. On the polymerized vegetable oil column, eight peaks were obtained. Such separation indicates the potentiality of these materials. However, separation was still far from complete. For example, one of the major peaks contained all triglycerides with a total of seven double bonds as well as those triglycerides with six double bonds and a two-carbon shortening in a single fatty acid. This behavior is very similar to that observed previously with free fatty acids and methyl esters (52). The application of other separation methods to mixtures of triglycerides probably will result in separation patterns similar to those they effect with the free acids.

IV. ANALYSIS

Chemical and Physical Methods. Chemical methods for the analysis of glycerides are, with one exception, unsatisfactory. The 1-monoglyceride content of a lipid can be determined accurately by oxidation with periodate and subsequent titration with sodium arsenite. Since free glycerol is oxidized also, it is removed by a preliminary water wash. Phospholipids also react with periodate and must be removed prior to the oxidation. The methods for determining the free glycerol and the 1-monoglyceride content of a lipid have been described in detail (95).

Periodate oxidizes compounds that have adjacent hydroxyl groups, such as 1-monoglycerides, but will not react with 2-monoglycerides or diglycerides. Many biological reactions involve 2-monoglycerides as an intermediate. Moreover, at chemical equilibrium, 12% of the monoglycerides will have the 2-configuration. Therefore, in order to obtain a true estimate of monoglyceride content, it is necessary to have a means of measuring the 2-monoglyceride as well as the 1-monoglyceride in a lipid. The demonstration by Martin (43) that treatment with perchloric acid of a lipid sample containing monoglycerides causes isomerization to an 88 to 12 mixture of 1- and 2-monoglycerides forms the basis of such an analysis. The applicability of this method to biological systems has been established (96). The method consists of determinations of the 1-monoglyceride content before and after isomerization of the sample with perchloric acid. The value obtained before isomerization gives the 1-monoglyceride level. The value obtained after isomerization is multiplied by the factor 1.15; the product gives the total monoglyceride content. The difference between the two values represents the 2-monoglyceride content. Since Martin's original observation, a number of minor changes in his original method have been suggested (45, 97).

Numerous chromatographic procedures for the quantitative determination of monoglycerides have been proposed. Our experience has been mainly with the method of Quinlin and Weiser (28), which uses silica gel columns. The results obtained by this method are almost as accurate as those obtained by chemical analysis and in some circumstances the chromatographic method is more rapid. Chromatography, however, yields only total monoglyceride values and does not furnish separate values for the 1- and 2-isomers.

It should be noted that the method of Lambert and Neish (78) for determining free glycerol involves a periodate oxidation step. As a consequence, any 1-monoglyceride that is present will be oxidized also

and reported as free glycerol. This problem will exist whenever samples are analyzed without preliminary removal of the lipid component. Korn (98) has pointed out this possible source of error in the method he has reported. Moreover, this method includes a preliminary treatment of the sample with sulfuric acid. This will cause partial isomerization of any 2-monoglycerides that may be present to 1-monoglycerides.

No direct method for the chemical determination of diglycerides exists at the present time. The usual procedure is to determine the number of free hydroxyl groups in a lipid (95), to correct this value for those due to monoglycerides (the level of this constituent is separately determined by the method of Martin (43)), and to calculate the diglyceride content from the remaining hydroxyl groups. The shortcomings in the method are apparent. Other nonchemical methods have been suggested but their reliability in the quantitative determination of diglycerides has not been fully established. These methods include infrared spectroscopy (99), differential thermal analysis (24), thin-layer chromatography (25), and a variety of column chromatographic methods (91). Again we have found silica gel column chromatography (28) to yield fairly accurate results. A direct, chemical method for determining the diglyceride content of a fat, particularly the separate measurement of the 1,2- and 1,3-diglycerides, is still needed.

The specificity of pancreatic lipase for the hydrolysis of the primary esters of glycerides forms the basis of a method for determining the structure of diglycerides. The isolated diglyceride is acylated with a "marker" fatty acid and the resulting triglyceride is hydrolyzed with pancreatic lipase. The proportion of "marker" fatty acid in the fatty acids present as monoglycerides after digestion is a direct measure of the amount of 1,3-diglyceride in the original sample (9).

Distribution of Fatty Acids in Glycerides. The complete characterization of a mixed acid di- and triglyceride includes the establishment of the position a fatty acid occupies in the glyceride molecule. In a few limited cases, it has been possible to do this by X-ray diffraction of the fully hydrogenated derivative. Mattson and Beck (100) demonstrated that pancreatic lipase specifically hydrolyzes the primary esters of glycerides and suggested that this specificity afforded a tool for determining the positional distribution of fatty acids. A detailed description of the method to be used with mono-, di-, and triglycerides has been published by Mattson and Volpenhein (9). This technique has been used in establishing the fatty acid distribution in the triglycerides of a number of animal and vegetable fats (1, 101). It should be noted that this procedure gives only the distribution of fatty acids between the primary and secondary positions. It does not yield information on the structure of individual triglycerides in a fat made up of a mixture of triglycerides.

The other type of structural studies that have been carried out seek to establish the content of trisaturated ($G\text{-}S_3$), disaturated-monounsaturated ($G\text{-}S_2U$), monosaturated-diunsaturated ($G\text{-}SU_2$), and triunsaturated ($G\text{-}U_3$) glycerides in natural fats. For many years these fractions have been determined by using a combination of crystallization and chemical oxidation. The details of this procedure have been reported by Hilditch (102). Recently some of the newer isolation techniques have been applied to this problem. Since the naturally occurring triglycerides are difficult to fractionate, the procedure used by Youngs (103) is to oxidize the unsaturated fatty acids of the triglycerides. This breaks the carbon chain of the unsaturated fatty acid at the double bond nearest the carboxyl group but does not break the ester bond. The presence of this short-chain acid, azelaic acid in most fats, in the triglycerides allows fractionation of the triglycerides by column chromatography. The separated triglycerides are then hydrolyzed with pancreatic lipase and the products identified by gas-liquid chromatography. In this manner, one determines not only the triglyceride type but also the isomeric form. For example, the content of G-SUS and G-USS are each determined. Privett and Blank (25) obtain the same information by separating the oxidation products by thin-layer chromatography. In neither instance is it possible to differentiate among the usual unsaturated fatty acids because all of these yield the same nine carbon dicarboxylic acids on oxidation.

In spite of these recent advances, the complete elucidation of the structures of the various triglycerides that are found in natural fats still is not possible. The problems are many, since a fat that contains only five fatty acids could consist of 75 triglycerides of different structures.

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