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The use of pancreatic lipase for determining the distribution of fatty acids in partial and complete glycerides

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SUMMARY

The method of digestion with pancreatic lipase for determining the position of fatty acids in triglycerides has been modified to increase its accurary and ease of applicability. Moreover, by acylation of partial glycerides with a "marker" fatty acid, the method can be used for determining the structure of mono- and diglycerides. Evidence is presented which demonstrates that acylation of partial glycerides with fatty acid chlorides can be carried out without causing rearrangement of the partial glycerides.

The demonstration that pancreatic lipase hydrolyzes specifically the fatty acids esterified with the primary hydroxyl groups of glycerol (1) served as the basis for a method to determine the distribution of fatty acids in a triglyceride (2, 3, 4). Considerable experience with this method has led to a number of refinements in the technique. Since interest in the chemistry and metabolism of glycerides of specific structures has become widespread, it seems desirable to report these refinements in technique. Moreover, by acylation of partial glycerides and hydrolysis of the resulting triglyceride with pancreatic lipase, it is also possible to determine the structure of mono- and diglycerides.

METHODS

Determining the Structure of Triglycerides. The steps in determining the structure of a triglyceride are: (*a*) digestion of the triglyceride with pancreatic lipase, (*b*) isolation of the monoglycerides from the products of digestion, and (*c*) determination of the fatty acid composition of these monoglycerides and the original triglyceride. Since the initial steps in the hydrolysis of a triglyceride by pancreatic lipase are the splitting-off of the fatty acids esterified with the primary hydroxyl groups of glycerol, the fatty acids present in the monoglycerides after the digestion will be those that occupied the 2-position in the triglyceride. The hydrolysis of the primary ester groups is random, showing neither fatty acid specificity (2) nor stereospecificity (5).

The conditions of digestion have been somewhat simplified from that originally reported (1). As presently carried out, the digestion mixture consists of 0.5 g of triglyceride, 9.0 ml of 1.0 M tris (hydroxymethyl) aminomethane adjusted to a pH of 8.0, 0.5 ml of a 45% aqueous solution of calcium chloride, 0.2 ml of a 1% aqueous solution of bile salts,[1] and 80 mg pancreatin[2] suspended in 1 ml of tris buffer. The pancreatin suspension is prepared immediately before use. The digestion is carried out at 40° with continuous rapid agitation. Digestion is continued, usually from 15 to 30 minutes, until approximately 60% of the esterified fatty acid has been hydrolyzed. The time of digestion necessary to obtain this degree of hydrolysis is determined with each batch of pancreatin.

At the end of the hydrolysis period 5 ml of dilute hydrochloric acid 1:1 (v:v) and 15 ml of ethanol are added. The lipids are then recovered by extraction with ethyl ether. The ethyl ether solution is washed three times with water, dried with sodium sulfate, and the solvent removed under vacuum. The resulting lipids are dissolved in benzene. The monoglycerides are isolated from these by chromatographing on silica gel by the method of Quinlin and Weiser (6), except that

[1] Bile Salts No. 3. Difco Laboratories, Detroit, Mich.
[2] Lipase (Steapsin). Nutritional Biochemical Corp., Cleveland, Ohio.

300 mm × 10 mm columns containing 10 g of silica gel are used. The fractions are eluted with 90 ml portions of solvent. The monoglyceride fraction is obtained from the column in solution in ethyl ether and contains about 5% of free fatty acid (FFA). To remove these FFA, an ethyl ether slurry containing approximately 5 g of Dowex 2-X8, 20-50 mesh anion exchange resin in the hydroxyl form is added. After filtering, the solvent is removed under vacuum. The fatty acids of the monoglyceride fraction and the original triglyceride are characterized by appropriate methods. The weight per cent of a particular fatty acid esterified with the 2-position will be equal to:

$$\frac{\text{per cent of the particular fatty acid in the monoglyceride formed}}{\text{per cent of the particular fatty acid in the original triglyceride} \times 3} \times 100.$$

During enzymatic hydrolysis, some of the fatty acids esterified in the 2-position of the triglyceride are split off. Whether this is due to an actual hydrolysis at the 2-position or whether the fatty acid migrates to one of the primary hydroxyl groups prior to hydrolysis is not known. Such splitting is indicated by the results shown in Table 1. Here a sample of cottonseed oil was hydrolyzed for various time periods and the iodine values of the FFA and of the monoglycerides were determined. The iodine value of the monoglyceride fatty acids remained constant but there was a gradual increase in the iodine value of the FFA. This increase in iodine value is the result of the splitting off of the acids esterified at the 2-position of glycerol, since in cottonseed oil the fatty acids occupying the 2-position are more unsaturated than those in the 1- and 3-positions (3).

TABLE 1. IODINE VALUE OF THE MONOGLYCERIDES AND FREE FATTY ACIDS RELEASED FROM COTTONSEED OIL BY PANCREATIC LIPASE AS A FUNCTION OF TIME

Time of Hydrolysis	Fatty Acids of the Monoglyceride	Free Fatty Acids
minutes		
15	154.9	87.8
20	155.4	92.1
30	155.0	93.5

Determining the Structure of Partial Glycerides. The principle of this determination depends on: (*a*) esterifying the partial glyceride with a "marker" fatty acid, (*b*) digesting the resulting triglyceride with pancreatic lipase, (*c*) isolating the monoglycerides from the products of digestion, and (*d*) determining the content of marker fatty acid in the monoglycerides resulting from digestion with pancreatic lipase. The enzymatic digestion and the isolation of the monoglycerides formed are carried out as already described in the section on Determining the Structure of Triglycerides. The marker fatty acid found in the monoglycerides present after digestion with pancreatic lipase will be proportional to the free hydroxyl groups in the 2-position of the original partial glycerides, with allowance being made for differences in molecular weight among the fatty acids. The marker fatty acid can be an isotopically labeled fatty acid or any fatty acid not present in the original partial glyceride. The method chosen for determining the content of marker fatty acid will depend on the nature of the fatty acids present in the original partial glyceride and the marker fatty acid used. For example, if the partial glyceride contains only saturated fatty acids, an unsaturated acid can be used as the marker, in which instance simply determining the iodine value of the monoglycerides formed by lipase hydrolysis will suffice.

The acylation of the partial glycerides with the marker fatty acid is carried out as follows. Approximately 0.6 g of monoglyceride or 0.9 g of diglyceride is dissolved in 10 ml of water-washed, distilled, and dried chloroform. An amount of pyridine equal, on a molar basis, to the amount of acid chloride to be used is added, followed by one and one-half times the amount of acid chloride which is necessary for esterification. The mixture is allowed to stand at room temperature for 3 days. As will be shown subsequently, the partial glycerides are completely esterified with no isomerization having taken place. The reaction products are dissolved in ethyl ether, washed twice with water, three times with 1% aqueous hydrochloric acid, four times again with water, dried with sodium sulfate, and finally the solvent is removed under vacuum. The washing steps are for the purpose of removing the pyridine and converting any remaining acid chloride to the free acid.

The lipids recovered from the acylation procedure consist of about 85% triglyceride and 15% FFA. The yield of triglyceride is from 90% to 100% of theory. The complete removal of this amount of FFA would be laborious. However, no attempt is made to remove this, since further steps in the procedure depend only on the glyceride fraction. To demonstrate that this residual FFA does not interfere with the method, samples of

1,2- and 1,3-dipalmitin[3] were esterified by this procedure. A portion of the resulting triglycerides was purified further by crystallization to an acid value of less than 1. The crude and recrystallized triglycerides were hydrolyzed with pancreatic lipase and the distribution of the marker fatty acid determined. In this instance, oleic-1-C^{14} acid was used. Samples were analyzed by counting in a Tri-Carb liquid scintillation spectrometer. The results are given in Table 2. It is apparent that this presence of FFA in the crude triglycerides did not alter the results.

TABLE 2. COMPARISON OF THE STRUCTURE OF DIGLYCERIDES AS DETERMINED ON CRUDE OR RECRYSTALLIZED TRIGLYCERIDES RESULTING FROM THE ACYLATION OF THE DIGLYCERIDES

Diglyceride Source	Per Cent 1,3 Isomer as Determined on:	
	Crude Triglycerides	Recrystallized Triglycerides
1,3-Dipalmitin	91	92
1,2-Dipalmitin	5	3
Mixture 1,2- and 1,3-Dipalmitin	60	61

For this method to be successful, it is necessary that there be complete acylation of the partial glycerides with the marker fatty acid and no migration of the fatty acid groups on the glyceride. Acylation was shown to be complete in that the glycerides isolated at the end of the acylation step had a hydroxyl value of zero, and acylation with radiotagged fatty acid yielded a glyceride which contained an amount of tagged acid theoretically correct for the formation of triglycerides.

Fischer and Baer (9) have reported that migration of fatty acids does not occur during acylation of monoglycerides with fatty acid chloride. Their conclusion was based on an unchanged optical activity and the content of 1-monoglyceride following exposure of an optically active 1-monoglyceride to pyridine. To demonstrate that this is also true of diglycerides, the following study, in which acylation occurred in two steps, was carried out. Samples of 1,2- and 1,3-dipalmitin were treated separately with acid chloride according to the procedure described here, except that for each mole of diglyceride one-half mole of carboxyl-tagged oleoyl-1-C^{14} chloride was added. Since the amount of acid chloride added was insufficient for complete acylation of the diglycerides, there was maximum opportunity for acyl migration to occur. After 72 hours of exposure to these conditions, 1 mole of fatty acid chloride was added and the reaction allowed to proceed for an additional 72 hours to completion. The resulting triglyceride was isolated and the location of the marker fatty acid determined in it. Other samples of the diglycerides were treated by the method which is being proposed here, where acylation takes place in one step. The analytical values obtained are given in Table 3. The amount of marker fatty acid found in the 2-position of the triglyceride was the same regardless of whether acylation was carried out in one or two steps. Thus acyl migration does not occur during the acylation step. Once the diglycerides or monoglycerides have been converted into triglycerides, there is no further possibility of acyl migration.

TABLE 3. DISTRIBUTION OF MARKER FATTY ACIDS IN DIGLYCERIDES FOLLOWING ACYLATION WITH FATTY ACID CHLORIDE IN ONE OR TWO STEPS

Diglyceride Acylated	Acylation Condition	Marker Fatty Acid in 2-Position of Triglyceride
		per cent
1,2-Dipalmitin	One step	6
1,2-Dipalmltin	Two steps	7
1,3-Dipalmitin	One step	95
1,3-Dipalmitin	Two steps	95

RESULTS

Application of the Method to Monoglycerides. The results obtained on applying this procedure to monoglycerides[4] are given in Table 4. In this instance linoleic acid was used as the marker fatty acid, since none of it was present in the monoglycerides studied. For comparative purposes, the values obtained by the periodic acid method (11) are given. It will be noted that the values obtained by the two methods are in good agreement. The lipase method will probably have only limited application to monoglycerides since the periodic acid method is much easier to carry out. How-

[3] The 1,2-dipalmitin was obtained by the incomplete acylation of 2-monopalmitin, prepared by the method of Martin (7), and isolation of the diglyceride by chromatographing on silica gel (6). The 1,3-dipalmitin was prepared by the method of Baur and Lange (8).

[4] The 1-monoglyceride was synthesized from isopropylideneglycerol (10) and the 2-monoglyceride was prepared by the method of Martin (7).

ever, the values in this table have been reported for the purpose of allowing a comparison of the new method with one that is already established.

TABLE 4. STRUCTURE OF MONOGLYCERIDES AS DETERMINED BY THE PERIODIC ACID AND LIPASE METHODS

Monoglyceride Source	Per Cent 1-Monoglyceride as Determined by:	
	Periodic Acid Method	Lipase Method
2-Monopalmitin	2	3
2-Monopalmitin	10	7
1-Monopalmitin	98	98
Mixture of 1- and 2-monoglycerides	88	86

Application of the Method to Diglycerides. The structures of three different samples of 1,2- and 1,3-diglycerides determined by this procedure are given in Table 5. The marker fatty acid used in these instances was oleate-1-C^{14}. Unfortunately, there is no other method reported for determining the structure of diglycerides to which these values could be compared. Because of the ease with which one isomer of a diglyceride can be converted to the other, it is difficult to retain a diglyceride in a single isomeric form. Hence, the fact that an old sample of 1,2-dipalmitin was found to consist of 49% of the 1,3 form, and none of the other diglycerides were entirely of their supposed structure, does not mean that the method of analysis is in error by these amounts. The critical test of the procedure is the results obtained on the mixtures of 1,2- and 1,3-diglycerides. On the basis of the weight of each diglyceride used in making up the mixture and the values obtained on the individual components, the theoretical values for the 1,3-diglyceride content was calculated. The values obtained were within 5% of those expected.

DISCUSSION

It is impossible at this time to be certain of the error involved in this method. Since triglycerides of supposedly known structure are synthesized from mono- and diglycerides, their structure can be no more certain than that of the partial glycerides from which they are prepared. Thus, the ease with which mono- and diglycerides isomerize makes uncertain the absolute purity of many triglycerides whose preparation has been reported. This ready isomerization of partial glycerides also precludes the use of mono- and diglycerides as analytical standards. Hence, methods which do not rely on analytical standards must be used to determine the reliability of this method. In the case of monoglycerides, an independent method is available. A comparison of the results obtained by the two methods, as shown in Table 4, indicates the error by the lipase method to be quite small. The results obtained on the mixtures of diglycerides (Table 5) again point to a relatively small error. It is probable that the values obtained by the lipase method are within 5% of the true value. Where the original glyceride contains fatty acids less than 12 carbon atoms in chain length, the presence of esterase in the pancreatin may interfere with this method.

This method can be used also for determining the distribution of fatty acids in a diglyceride of mixed fatty acid composition. The fatty acid composition of the diglyceride is determined by one of the standard methods. Using the methods described here, the diglyceride is acylated with a marker fatty acid, digested with pancreatic lipase, and the resulting monoglycerides characterized. The marker fatty acid is used to determine the position of the free hydroxyl group in the original diglyceride. The proportion of a particular nonmarker fatty acid in the monoglycerides resulting

TABLE 5. STRUCTURE OF DIGLYCERIDES AS DETERMINED BY THE LIPASE METHOD

Diglyceride Source*	Per Cent 1,3-Diglyceride	
	Theory*	Found†
1,3-Dipalmitin		94, 96, 100
1,2-Dipalmitin		3, 5, 6
Mixture of Above 1,3- and 1,2-Dipalmitin	50	49
1,3-Dipalmitin		94, 96, 100
1,2-Dipalmitin		12, 12, 15
Mixtures of Above 1,3- and 1,2-Dipalmitin	67	67
	28	27
	46	50
1,3-Dipalmitin		92, 95, 97
1,2-Dipalmitin, old sample		49, 49
Mixtures of Above 1,3- and 1,2-Dipalmitin	71	73
	64	65
	88	89
	89	92

* Theoretical values for the mixtures of diglycerides are based on the proportions used in making these mixtures and the values obtained on the individual components by the lipase method.

† Each figure reported is that of a separate analysis.

from lipase digestion will be proportional to the amount in the 2-position of the original diglyceride.

A sequence of reactions similar to those reported here has been used by Tattrie (12) for determining the location of fatty acids in lecithin. The initial step he employs is the splitting of phosphorylcholine from lecithin. His subsequent steps are similar to those reported here for a diglyceride, except that he uses the composition of the fatty acids freed by lipase hydrolysis to determine the distribution of the fatty acids in the glyceride. Although he is careful to allow the hydrolysis to proceed only to the diglyceride stage, there is always the danger that some of the fatty acids esterified with the 2-position will appear as FFA. In the method described in the present paper, the enzymatic hydrolysis of the triglycerides is carried out in the presence of a sufficient excess of calcium so that the fatty acids which are freed are immediately converted to insoluble calcium soaps. Because of this, these fatty acids cannot take part in esterification reactions. Thus the monoglycerides which are present at the end of the enzymatic digestion have been formed solely by the successive hydrolysis of triglycerides yielding 1,2-diglycerides then 2-monoglycerides. If a portion of the 2-monoglycerides are in turn hydrolyzed, it will not influence the results, since only the monoglyceride fraction is analyzed. The advantage of using the monoglyceride rather than the FFA fraction is apparent.

The method for determining the structure of partial glycerides is not directly applicable to a mixture of mono- and diglycerides; one must first separate them by a suitable method. This isolation, or any other treatment of mono- and diglycerides, must be carried out under rigorously controlled conditions because of the ease with which these partial glycerides isomerize. For example, even a brief exposure to heat will bring both mono- and diglycerides to their normal equilibrium mixture (13).

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