The Pancreatic Hydrolysis of Natural Fats. III. The Influence of the Extent of Hydrolysis on Monoglyceride Composition

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Abstract

Samples of lard and illipé butter have been hydrolyzed with pancreatic lipase for varying lengths of time, and the products estimated and analyzed. The composition of the monoglycerides from both fats, and of the tri- and diglycerides of illipé butter, were independent of the degree of hydrolysis; but the tri- and diglyceride fractions of lard became progressively more saturated with increasing hydrolysis. These results provide evidence for the sort of fatty acid distribution in fats envisaged by VanderWal. Representation of the results by a theoretical expression suggests that the rates of hydrolysis of the tri- and diglycerides are similar. It is concluded that analysis of the monoglycerides provides a better estimate of the distribution of fatty acids in natural fats than the analysis of the free fatty acids.

Introduction

THE POSITIONAL specificity of pancreatic lipase has been utilized by a number of workers for the investigation of the distribution of fatty acids in natural fats (1,2,3,4,5,6,7,8). This enzyme brings about the preferential hydrolysis of the fatty acid residues occupying the outer (i.e. the 1 and 3) positions of the triglycerides; and when half of the acids have been liberated, the principal product is a mixture of 2-monoglycerides.

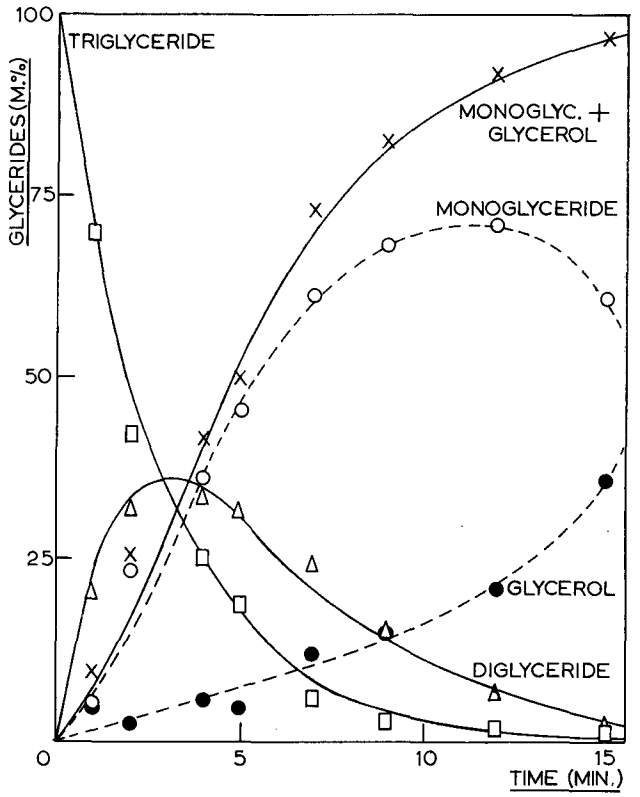

FIG. 1. Time course for the hydrolysis of illipé butter. Continuous line—theoretical curves calculated from expressions given in text. Broken line—drawn through experimental points.

Some authors (5,9) have used the composition of the free fatty acids to calculate the distribution in the original triglycerides; but Coleman and Fulton (7) have shown that appreciable amounts of glycerol may be formed in these hydrolyses, so that some of the free fatty acids must be derived from the 2-positions. For this reason, Coleman (8) has suggested that the composition of the fatty acids of the 1 and 3 positions is best calculated from the difference in composition between the original triglycerides and the resultant monoglycerides.

Savary, Desnuelle (10), and Coleman (11) have reported that where saturated and unsaturated acids occupy equivalent positions on a synthetic triglyceride molecule, the unsaturated acid is more readily removed, and the resulting diglyceride is more saturated than theoretical considerations would suggest. However, as Coleman (11) has pointed out, the positional specificity is paramount; confronted with a 1-saturated, 2-unsaturated diglyceride, the enzyme removes the saturated acid. The monoglyceride therefore has the expected composition and provides a reliable measure of the fatty acid composition of the 2-positions of the original triglyceride molecules.

In the case of natural fats, there remains the question of whether there is any selective hydrolysis of particular triglycerides. To test this, two fats have been subjected to pancreatic hydrolysis for varying lengths of time, and the products isolated and analyzed. When a completely interesterified fat is hydrolyzed, each glyceride fraction and the free fatty acids will have the same fatty acid composition; so to provide an adequate test of the influence of the extent of hydrolysis on the monoglyceride composition, it is necessary to employ fats which differ as markedly as possible from such a random fatty acid distribution. Several such fats are known. The two employed here were, illipé butter [one of the "Bassia fats" (12)], where departure from random distribution is in the direction of an excess of unsaturated acids in the 2-positions; and lard, where the converse is true.

Experimental

The fats used were commercially refined. For each hydrolysis the triglyceride fraction of approximately 1 g of the fat was obtained chromatographically (13). A 50 mg sample of each was saponified in alcoholic KOH, and after acidification the fatty acid composition determined by gas-chromatography (14). The remainder of each triglyceride fraction was then subjected to pancreatic hydrolysis at pH 8.5 in a 1.2 M NH_4Cl/NH_4OH buffer containing 2 ml of a 22% w/v $CaCl_2$ $6H_2O$ solution, and 0.1 ml of a 25% w/v bile salt solution.

Hydrolysis of illipé butter was carried out with 50 mg of a purified pancreatic lipase (15) at 37.5C: for the lard samples, 100 mg of the lipase were used, and the hydrolyses run at 45C. The reaction was

TABLE I
Fatty Acid Composition of Illipé Butter and of the Products of Hydrolysis

Glyceride	Hydrolysis		Fatty acid composition, M %						Mean mol wt
	Time min	Extent %	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic	
Initial & residual triglycerides (mean)	....	....	16.5	0.3	45.8	35.4	0.6	1.4	875
Diglycerides formed (mean)	....	....	11.7	....	36.9	49.3	1.0	1.1	616
Monoglycerides formed	1	14.8	1.9	....	2.5	93.8	1.8	....	....
	2	28.6	2.0	....	3.5	93.0	1.5	....	....
	4	40.6	1.7	....	3.2	94.1	1.0	....	....
	5	45.1	1.7	....	4.2	93.0	1.1	....	356
	7	60.9	1.5	....	3.0	93.5	2.0	....	....
	9	64.9	1.1	....	2.0	96.4	0.5	....	....
	12	70.4	2.4	....	4.5	92.4	0.7	....	....
	15	77.0	2.6	....	4.1	92.6	0.7	....	....

terminated by bringing the pH to 1, with 4*N* HCl, and the reaction mixture extracted and analyzed in the usual way (8).

Results

The fatty acid compositions of the tri-, di-, and monoglycerides of illipé butter were found to be independent of the extent of hydrolysis, within the limits of experimental error (±2%). Moreover, the composition of the residual triglycerides left after the hydrolysis was found to be the same as that of the original triglycerides. Hence average molecular weights were calculated for each of the glyceride fractions, and from these and the weights of each fraction, the molar composition of each reaction mixture was calculated. These compositions, plotted against time, are illustrated in Figure 1, and Table I gives the fatty acid compositions of the fractions.

When the lard reaction mixtures were analyzed, it was found that the saturated acid content of the residual triglycerides and of the diglycerides formed, increased with increasing hydrolysis (Fig. 3). However, as this change was largely due to an increase in stearic acid, with a corresponding decrease in oleic, the effect on the average molecular weight was insignificant. The composition of the monoglycerides showed no significant variation (Table II). Again therefore, molar compositions were calculated from mean molecular weights for each fraction; that for the diglycerides was calculated as 596. The progress of the hydrolysis of lard is illustrated in Figure 2.

For both series glycerol was calculated by difference. The extent of the hydrolysis was expressed as the percentage of fatty acids released, of the total combined fatty acids originally present.

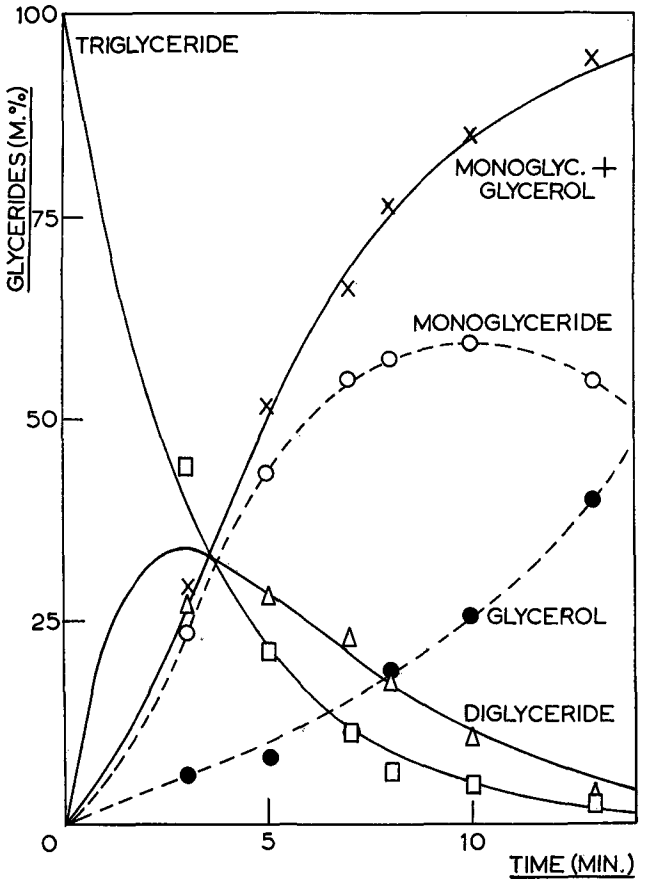

FIG. 2. Time course for the hydrolysis of lard. Continuous line—theoretical curves calculated from expressions given in text. Broken line—drawn through experimental points.

Discussion

The positional specificity of pancreatic lipase suggests that the complete hydrolysis of a triglyceride involves at least one isomerization step, in addition to the three hydrolytic steps, thus:

Triglyceride → 1,2-Diglyceride → 2-Monoglyceride ⇌ 1-Monoglyceride → Glycerol.

This system is too complex to treat theoretically, and in any case the heterogeneous character of the reaction mixture makes it difficult to assign precise significance to the rates measured. However, as Figures 1 and 2 illustrate, the progress of the hydrolysis may be adequately represented by the expressions for two consecutive reactions, if the final product is regarded as monoglyceride; i.e., by adding the monoglyceride and glycerol percentages together. Then:

$$\text{Triglyceride} \xrightarrow{k_1} \text{Diglyceride} \xrightarrow{k_2} \text{Monoglyceride}.$$

The concentrations of the three components at time t are given (16) by the expressions:

TABLE II
Fatty Acid Composition of Lard and of Monoglycerides Produced by Hydrolysis

Glyceride	Hydrolysis		Fatty acid composition, M %								Mean mol wt
	Time min	Extent %	Lauric	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Eicosenoic	
Initial triglyceride (mean)	....	....	0.9	1.7	28.0	4.6	19.8	39.0	4.3	1.7	859
Monoglycerides formed	3	30.2	....	4.8	65.8	8.7	5.8	13.0	1.9	....	....
	5	46.3	1.2	4.7	62.5	8.0	6.8	14.1	2.7	....	....
	7	55.3	2.2	4.3	65.0	7.8	6.1	12.9	1.7	....	....
	8	63.1	0.9	5.3	64.4	8.2	5.0	14.9	1.3	....	334
	10	68.7	0.7	6.0	62.6	10.0	6.6	13.2	0.9	....	....
	13	77.4	0.6	4.6	65.1	10.4	6.2	12.0	1.1	....	....

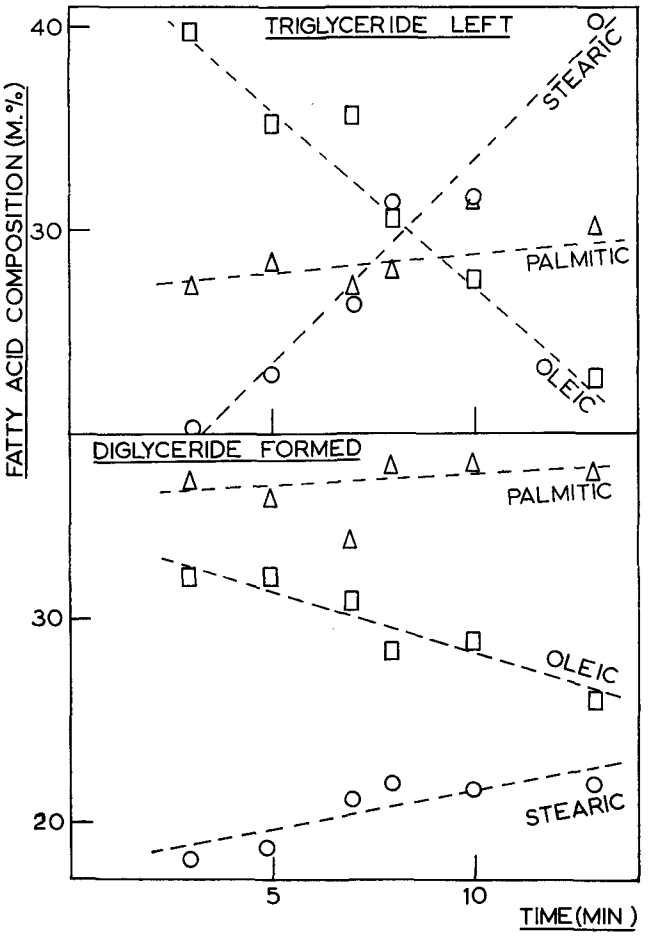

FIG. 3. Change of composition of residual triglycerides, and of the diglycerides formed, with increasing hydrolysis of lard.

$$(T)_t = (T)_o e^{-k_1 t}$$

$$(D)_t = (D)_o + \frac{k_1 (T)_o}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t})$$

$$(M)_t = (M)_o + (T)_o \left[1 - \frac{1}{k_2 - k_1} (k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right]$$

Where (T) = Triglyceride, (D) = Diglyceride, (M) = Monoglyceride concentrations.

The velocity constants k_1 and k_2 were estimated as 0.35 and 0.36 min^{-1} for illipé butter, and 0.31 and 0.36 min^{-1} for lard, respectively. Clearly these are merely overall rate constants, and represent the net result of a number of processes; the formation of the enzyme-substrate complex, its breakdown, and probably a diffusion process as well. The results resemble those obtained by Desnuelle et al. (17), but in the present hydrolyses glycerol appears at an earlier stage, particularly for the lard samples.

The results show that the composition of monoglycerides produced by the pancreatic hydrolysis of natural fats is independent of the extent of the hydrolysis. At first sight this appears paradoxical when, as in the case of lard, saturated acids accumulate in the residual triglycerides, and in the diglycerides formed with increasing hydrolysis. The explanation would appear to lie in the form of the fatty acid distribution for natural fats, proposed by VanderWal (18) and Coleman and Fulton (7). This supposes that the fatty acids of the 1 and 3 positions are randomly distributed with respect to the 2-positions. The triglycerides of such a fat may be considered as falling into classes, each class with a characteristic combination of fatty acid residues in the 1 and 3 positions. Since the rate of hydrolysis will depend principally on the acids of the outside positions, all members of such a class will be hydrolyzed at much the same rate, but the different classes will be hydrolyzed at different rates.

The point is perhaps best illustrated with an example. Thus the lard used here may be regarded as a fat containing 50.4% saturated acids (S), and 49.6% unsaturated acids (U). On hydrolysis it yields a monoglyceride containing 76.2% S and 23.8% U. The triglyceride composition may then be calculated in the usual way (8). The values of the six glycerides are given in Table III. It will be seen that the glycerides fall into three classes:

a. With two S residues in the outside positions.
b. With one S and one U.
c. With two U residues.

Since U will be more readily removed from the 1 and 3 positions than S, class c triglycerides will be hydrolyzed more rapidly than class b, and class b than class a. Initially the hydrolysis will be predominantly of class c glycerides, followed by class b and concluding with class a. Hence the triglycerides left will become increasingly saturated as hydrolysis proceeds.

Similarly the diglycerides formed will become increasingly saturated, since those with unsaturated acids in the 1-position will be more rapidly hydrolyzed than those with saturated acids in this position.

But the result of the fatty acid distribution envisaged is that the proportion of S in the 2-positions of each glyceride class is the same (76%); so each class will yield a monoglyceride of the same composition. Hence, the monoglyceride composition will remain constant throughout the hydrolysis, provided the monoglycerides are hydrolyzed much more slowly than di- and triglycerides.

In this example the fat is considered as consisting of two acids only, but clearly the same arguments apply where more acids, and hence glyceride classes, are considered.

The progressive change in composition of the residual triglycerides, and of the diglycerides, formed from lard, indicates that the free fatty acid composition varies with the extent of the hydrolysis. This conclusion is confirmed by the observation of Mattson and Volpenheim (19), who have reported a progressive change in the iodine value of liberated fatty acids. The early appearance of glycerol shows that some of the free fatty acids have been derived from the 2-

TABLE III

Glyceride Composition of Lard (VanderWal Distribution) Showing Monoglyceride Composition to Be Independent of Glyceride Class Hydrolyzed.

Glyceride class	Acids of 1 + 3 positions	Calculated glyceride composition, M %				% S in monoglycerides produced
		Glycerides yielding S-monoglycerides		Glycerides yielding U-monoglycerides		
		Glyceride	M %	Glyceride	M %	
a	S + S	SSS	10.7	SUS	3.3	10.7 ÷ (10.7 + 3.3) × 100 = 76
b	S + U	SSU	35.8	SUU	11.2	35.8 ÷ (35.8 + 11.2) × 100 = 76
c	U + U	USU	29.7	UUU	9.3	29.7 ÷ (29.7 + 9.3) × 100 = 76

positions of the triglycerides. For some fats at least, the free fatty acids do not, therefore, provide a satisfactory measure of the composition of the acids occupying the 1 and 3 positions of the triglycerides.

It must of course be remembered when using pancreatic lipase for the investigation of the fatty acid distribution of natural fats, that rates of hydrolysis for individual fatty acids are only comparable for the higher members of the series. Savary and Desnuelle (20) have reported that similar rates are obtained for lauric, and acids of greater molecular weights, whether saturated or unsaturated. Entressangles et al. (21) and Clément et al. (22) have clearly shown that the presence of short chain acids, particularly butyric, obscure the results of fatty acid distribution deduced from hydrolysis data. Entressangles has drawn attention (23) to the unsuitability of pancreatic hydrolysis for the investigation of such fats as milk fat, which contain appreciable amounts of butyric acid.

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Gossyverdurin: A Newly Isolated Pigment from Cottonseed Pigment Glands

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Abstract

The constituents of cottonseed pigment glands were fractionated by the use of column chromatography with DEAE cellulose ion exchanger and silicic acid, and a new green pigment was isolated. The acute oral toxicity of the new pigment was determined using rats as experimental animals. The LD-50 value obtained was 0.66 g/kg of body weight indicating that the new pigment which was named gossyverdurin is the most toxic of any cottonseed pigment so far reported. Gossyverdurin showed absorption maxima at 250, 370, and 560 mμ. Reaction with para-anisidine under the conditions used for the determination of gossypol gave an absorption peak similar to that obtained with gossypol indicating that the new compound is structurally related to gossypol. In addition a second peak at 342 mμ appears on reaction with para-anisidine indicating important structural differences between gossypol and gossyverdurin.

Introduction

IT HAS BEEN reported that cottonseed pigment glands, which are separated in an essentially unaltered condition from cottonseed kernels by a flotation process (1,2) retard the growth of chicks when the glands or products containing them are included in the diet (3,4). Oral administration of these glands in relatively large doses resulted in the death of rats, mice, guinea pigs, and rabbits (5).

Gossypol, a polyphenolic yellow pigment, is the principal component and gossypurpurin, a gossypol derivative, has been reported to be the most abundant secondary component of cottonseed pigment glands (6).

Eagle et al. (5) studied the relative toxicity of pure gossypol and a number of preparations of pigment glands using rats as experimental animals and reported that the glands were more toxic than an equivalent amount of gossypol, that the toxicity of different gland preparations was not proportional to their content of gossypol, and that toxicity decreased with an increasing content of gossypurpurin. They concluded: "The toxicity of cottonseed pigment glands is attributable to some component or components of the glands other than, or in addition to, gossypol and gossypurpurin." In these studies an acetone-soluble, water-soluble fraction was obtained from the glands which had an LD-50 value of approximately 700 mg/kg body weight, using rats as experimental animals.

Recently the reports of Eagle et al. to the effect that cottonseed pigment glands are more toxic than an equivalent amount of gossypol have been confirmed by El-Nockrashy, Lyman, and Dollahite (7). The purpose of the present communication is to report the isolation of a bright green colored pigment from cottonseed glands which is more toxic than gossypol. This new pigment, which is a gossypol related compound, has been named gossyverdurin.

Experimental

Fractionation of Cottonseed Pigment Glands. Cottonseed pigment glands were separated from rolled decorticated kernels by a flotation technique (8). Twenty-five grams of the pigment glands were extracted with 200 ml of acetone in a Waring Blendor for 5 min, and the mixture was filtered through a Buchner funnel. The residue was re-extracted with 200 ml, and finally with 50 ml of acetone. The com-