^{13}C Nuclear Magnetic Resonance Characterization of the Reaction Products of Lamb Pregastric Lipase-Catalyzed Hydrolysis of Tributyrylglycerol

Richard H. Barton and Charmian J. O'Connor*

Department of Chemistry, The University of Auckland, Auckland, New Zealand

ABSTRACT: Lamb pregastric lipase (LPGL), extracted from the tongue root and epiglottis of suckling lamb, has been used to catalyze the hydrolysis of emulsified tributyrylglycerol. Reactions were generally carried out at 35°C and initial pH 7.0. The speciation of the products of the reaction has been examined by ^{13}C nuclear magnetic resonance spectroscopy. Varying rates of reaction were produced by increasing the amount of enzyme added relative to the amount of lipid. A system rate parameter, $\Delta pH/\Delta t$, which is the change in pH (ΔpH) from time zero to the time when the sample was removed (Δt), has been developed. 1,2(2,3)-Dibutyrylglycerol and 2-monobutyrylglycerol have been identified as products of LPGL-catalyzed hydrolysis, while 1,3-dibutyrylglycerol and 1(3)-monobutyrylglycerol are products of uncatalyzed acyl transfer reactions.

JAOCS 75, 967–976 (1998).

KEY WORDS: Acyl transfer, diacylglycerols, enzyme catalysis, hydrolysis, monoacylglycerols, pregastric lipase, triacylglycerols, tributyrylglycerol.

Pregastric lipases (PGL), including lamb pregastric lipase (LPGL), have had a long involvement in ancient biotechnology, including milk-fat processing, some early cases of which are well-documented. Today, these usages continue on a more sophisticated and specific basis (1). Reviews have covered a large range of approaches and fields of study (2–4). The term "pregastric" is used in distinction to "gastric" to emphasize the source and nature of these enzymes. Detailed physiological investigation has provided much information on the exact position of secretion of PGL (5).

In ovine milk fat, all $C_{4:0}$ acids are at the *sn*-3 position. This position is the preferential location of the short-chain fatty acids of milk fat, with the remainder of its occupancy preferentially being $C_{18:n}$ ($1 < n < 3$) fatty acids. The shorter-chain fatty acids, from $C_{4:0}$ to $C_{10:0}$, together represent 15.9 mol% of the total fatty acids present, and of these shorter-chain acids, 79.7% are at the *sn*-3 position (6).

LPGL is the first enzyme involved in the digestion of ovine milk fat. Given the ancient evolutionary codevelopment of milk and PGL for effective milk processing in the suckling young, we may expect the milk-fat triacylglycerol (TAG) structure to mirror properties exhibited by PGL.

We have used PGL to catalyze the hydrolysis of monoacid TAG ($C_{4:0}$ to $C_{12:0}$) and maximal activity was obtained against tributyrylglycerol (TBG) (7,8). We also have made a preliminary investigation of the speciation of the products of LPGL-catalyzed hydrolysis of TBG and found clear evidence of uncatalyzed conversion of the 1,2(2,3)-diacylglycerol [1,2(2,3)-DAG] to the 1,3-isomer (9). In this investigation, we have extended this previous study and also have identified the presence of monoacylglycerols (MAGs) in the mixture of products. Partially acylated glycerols serve as precursors for structural molecules, such as phospholipids, and for membranes; act as signaling molecules; and are natural surfactants for pulmonary function. The structures that arise from the selectivities of the enzymes that dietary TAG encounter play a role in the pathways of absorption (10). This study confirms that, when TBG undergoes hydrolysis catalyzed by LPGL, 1,2(2,3)-DAG, 1,3-DAG, 1(3)-MAG, and 2-MAG are produced.

Development of a system rate parameter, and the use of varying rates of reaction, have enabled us to distinguish the products of hydrolysis from those of acyl transfer; and from primary product speciation, the positional preferences of LPGL could be delineated. Not all details of positional attack can be resolved by the use of standard ^{13}C nuclear magnetic resonance (NMR), and the inability to resolve the *sn*-1 from the *sn*-3 carbon, a stereospecific distinction, means that *sn*-1,2-DAG and *sn*-2,3-DAG, for example, are not differentiated. Other techniques, such as optical polarization–plane rotation, must be invoked to determine whether the preliminary LPGL-catalyzed hydrolysis is stereoselective. Such data now have been obtained (11), and these show that the first product of hydrolysis is exclusively the *sn*-1,2-DAG. This observation is rather unique because mammalian lipases are generally *sn*-1,3-selective (e.g., pancreatic lipase) or nonspecific (bile salt-stimulated human milk lipase). Nevertheless, for the purposes of this report, the product DAG will generally be referred to as 1,2(2,3)-DAG. However, we can distinguish *sn*-1,3- from 1,2(2,3)-DAG species because this requires positional information only, which is available from the NMR spectra of the reaction products.

*To whom correspondence should be addressed at Department of Chemistry, The University of Auckland, PB 92019, Auckland, New Zealand.
E-mail: cj.oconnor@auckland.ac.nz

EXPERIMENTAL PROCEDURES

Materials. Freeze-dried PGL from lamb (New Zealand Rennet Company Ltd., Eltham, New Zealand) was the unextended product and was generally used as supplied. In the preliminary experiment, it was used as the partially purified (12) product. The TBG (96 to 98% grade II), and reagent-grade buffers tris(hydroxymethyl)aminomethane (Tris) and 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) were from Sigma Chemical Co. (St. Louis, MO).

Product analysis reaction sequence. The following conditions pertain to Example I. The reaction emulsion contained 296 mg TBG and 2.5 mg partially purified LPGL (specific activity, 23.3 μmol free fatty acid min^{-1} mg^{-1}, 40°C, pH 6.5, 8.5 mM TBG, equivalent to 11.6 mg crude enzyme), which were added to 50 mL 100 mM Tris/HCl, at initial pH (pH_i) 7.04, 37°C. After initial sonication to form the emulsion, the mixture was stirred throughout the reaction. The method for Examples II–VI was similar, but some experimental details, especially the amount of enzyme added and the nature of the buffer, differed. The conditions for Examples II–VI are given in Table 1. All reactions were carried out in the absence of emulsifier.

The pH was monitored throughout the reaction, and at intervals 15-mL aliquots were removed for subsequent ^{13}C NMR analysis. The reaction was quenched by fast mixing with an approximately equal volume of cool $CHCl_3$. If necessary, the mixture was subjected to brief centrifugation, and the phases were then separated with a Pasteur pipette. Further $CHCl_3$ washes were employed and the $CHCl_3$ phase was pooled. This extract was then subjected to cool rotary evaporation (~20 mm Hg, 15–20°C), and the reaction products were transferred to $CDCl_3$ for ^{13}C NMR analysis. This method was exhaustive for TAG and DAG and recovered more than 90% of the MAG.

^{13}C NMR analysis and quantitation. The ^{13}C spectra were obtained by using $CDCl_3$ solutions and a carefully shimmed Bruker AC200 spectrometer (Rheinstetten, Germany). A Bruker AM400 instrument was also occasionally used. Long scan times were employed when required. Data were typically acquired at 50 MHz over 12.5 kHz into 32768 data points, and exponential multiplication was carried out with a reduced line broadening of 1 Hz prior to applying the Fourier transform. Spectra were referenced to $CDCl_3$. Species were identified by the patterns of their shifts and their intensities.

Although the shift values for acylglycerols are not precisely reproducible (6), the differences between shifts of carbons of similar environment within a species are more nearly so (13). For TAG and partial acylglycerols with longer-chain fatty acids, more data are available. However, chainlength does not affect greatly the spine carbon shifts, and so a wide selection of literature is indicative of shift values for spine signals for each of the positionally possible acylglycerol species (13–15). Each TAG and its derived DAG and MAG species, depending on their internal symmetry, have a set of shifts of defined relative intensities, which further aid peak and species identification, especially in spectra of complex mixtures. Typical values for the spine carbons of glycerol and its butyryl derivatives, obtained from a mixture of the co-present species, are given in Table 2. Assessment of mole percentage composition for each species was typically by peak height summation of appropriate sets of glycerol spine carbon resonances in the 60–74 ppm shift range, and extensive verification of this quantitation was carried out. This included cross-comparison with integration on the AM400, as well as verification that the pulse repetition delay was adequate. Additions were made to mixtures to ensure that the correct mole ratios were given over a range of compositions. Comparative quantitations performed in the absence of nuclear Overhauser effect (NOE) enhancement showed that there was no differential NOE enhancement of intensity for the nuclei of any species. Spectra were hence acquired with this enhancement to reduce the acquisition time required for satisfactory signal-to-noise ratios.

A set of ^{13}C NMR spectra for a reaction sequence (from the restricted region of quantitation) is shown in Figure 1. The profile illustrates typical reaction products, but the kinetic profile for this example is complex due to the addition of two further masses of crude enzyme during the reaction. The use of ^{13}C NMR for quantitating lipase activity in emulsion systems previously has been described (16).

TABLE 1
Experimental Conditions for LPGL-Catalyzed Hydrolysis of TBG[a]

	Example				
Condition	II	III	IV	V	VI
BTP (mM)	158	200	200	197	223
LPGL[b] (mg mL^{-1})	0.615	3.16	6.30	12.1	27.0
TBG (mM)	28.7	34.5	33.9	42.4	56.0

[a]35°C, pH_i ~7.0. LPGL, lamb pregastric lipase; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; TBG, tributyrylglycerol.
[b]Crude enzyme, specific activity 5.5 μmol free fatty acid min^{-1} mg^{-1} (40°C, pH 6.5, 8.5 mM TBG).

RESULTS AND DISCUSSION

Before discussing the results, it is necessary to address the question of whether acyl migration might have occurred during workup of the lipolysis samples for NMR analysis.

Traditionally, exhaustive lipid extractions from biological samples are carried out by Folch (17) or modified Folch pro-

TABLE 2
^{13}C Nuclear Magnetic Resonance Shifts[a] for the Spine Carbons of Glycerol and Its Butyryl Derivatives

Species	*sn*-1C	*sn*-2C	*sn*-3C	Intensity
1,2,3-TAG	61.9	68.8	61.9	2:1
1,2(2,3)-DAG	61.2[b]	71.9	62.1[b]	1:1:1
1,3-DAG	64.8	67.9	64.8	2:1
1(3)-MAG	63.2[b]	70.0	64.9[b]	1:1:1
2-MAG	61.6	74.7	61.6	2:1
Glycerol[c]	63.0	72.5	63.0	2:1

[a]Bruker AC200 (Rheinstetten, Germany), $CDCl_3$, δ in ppm. TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol.
[b]These assignments may be reversed.
[c]Pure glycerol as two-phase mixture with $CDCl_3$.

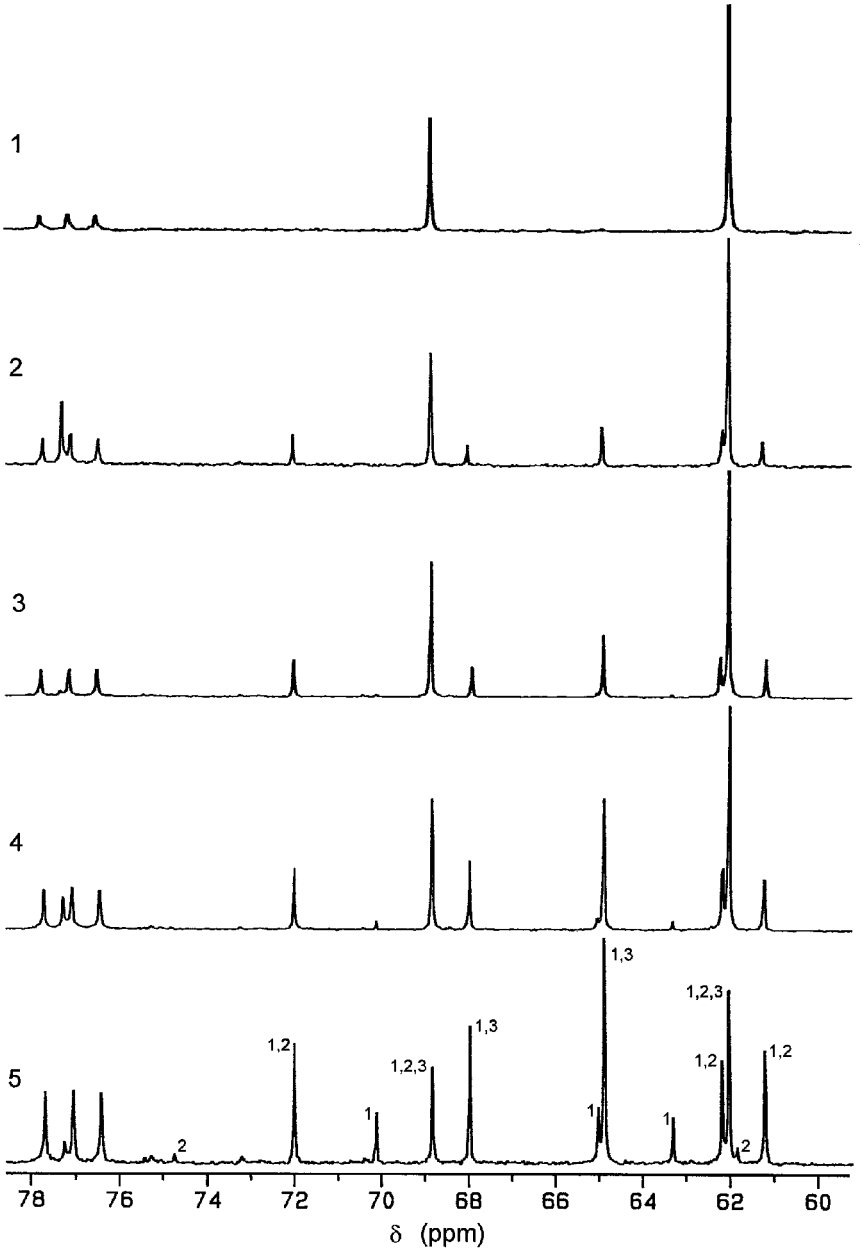


FIG. 1. ^{13}C nuclear magnetic resonance (NMR) spectra of a sample sequence from the lamb pregastric lipase (LPGL)-catalyzed hydrolysis of 716 mg tributyrylglycerol (TBG) in 70 mL of 200 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP): 1, TBG (38 mg crude enzyme added at time $t = 0$ and a further 16 mg at $t = 26$ min); 2, $t = 47$ min; 3, $t = 81$ min; 4, $t = 126$ min (15 mg crude enzyme added at $t = 161$ min); 5, $t = 180$ min. Peaks are identified by numbers in panel 5: 1,2,3 = TBG; 1,2 = 1,2(2,3)-diacylglycerol (DAG); 1,3 = 1,3-DAG; 1 = 1(3)monoacylglycerol (MAG); 2 = 2-MAG. Panel 1 represents the spectrum of unreacted TBG prior to enzyme addition.

cedures. The Bligh and Dyer (18) procedure, as modified from Folch *et al.* (17), employs a mixture of $CHCl_3$/MeOH (2:1 vol/vol) in quantity sufficient to give a homogeneous liquid phase by absorbing all the water in the sample. Solids are removed, and the addition of further $CHCl_3$ and water then yields a two-phase system, in which the $CHCl_3$-rich phase partitions almost quantitatively the lipid species present.

The use of Folch-type methods was excluded in this work because of the large volumes of water in the sample. The important choice of $CHCl_3$ alone ensured complementarity to NMR analysis and offered an optimal balance of simple, rapid, and quantitative extraction of the desired TAG and DAG species. Moreover, control experiments revealed that no isomerization took place in either $CHCl_3$ or $CDCl_3$. Long-

term storage (up to 12 mon at –20°C) in batches of varying TAG, DAG and MAG composition, including DAG present as more than 80% 1,2(2,3)-isomer, showed no change in composition (or optical activity). Moreover, sample mixtures in $CDCl_3$ held at 50°C for periods of over a week showed no evidence of isomerization.

Because the extraction procedure was rapid and the reactivity in wet $CHCl_3$, even at elevated temperatures, was examined and found to be absent, the NMR spectra reflect the true composition of the reaction sample before extraction. Enzyme is excluded in the extraction phase.

Figures 2–5 show the results for Examples I–IV, respectively. In each figure, the upper plot shows the decrease in pH as the reaction proceeded, and the lower plot shows the mole percentage composition of the sampled mixtures. Table 3 shows corresponding data for percentage composition for Examples V and VI and the pH at the time the reaction was quenched.

Example I. When first examined, the small excess (at 26 min) of the 1,2(2,3)-isomer of the DAG in Example I (Fig. 2) was considered a potential artifact of spectral noise. Subsequent investigation has shown that this small surplus of 1,2(2,3)-over 1,3-isomer was real, as was the later significant reversal (at 189 min) of this ratio. For this reaction sequence, the rise and fall of the proportion of 1,2(2,3)-DAG conform to first-order conversion kinetics in the reaction TAG → 1,2(2,3)-DAG → 1,3-DAG (+MAG), treated as a series first-order reaction A → B → C (14). In this model, the amount of B as a function of time is controlled by two first-order rate constants, covering formation of B and its removal. The values of the rate constants were 0.57 h^{-1} (formation) and 0.30 h^{-1} (removal).

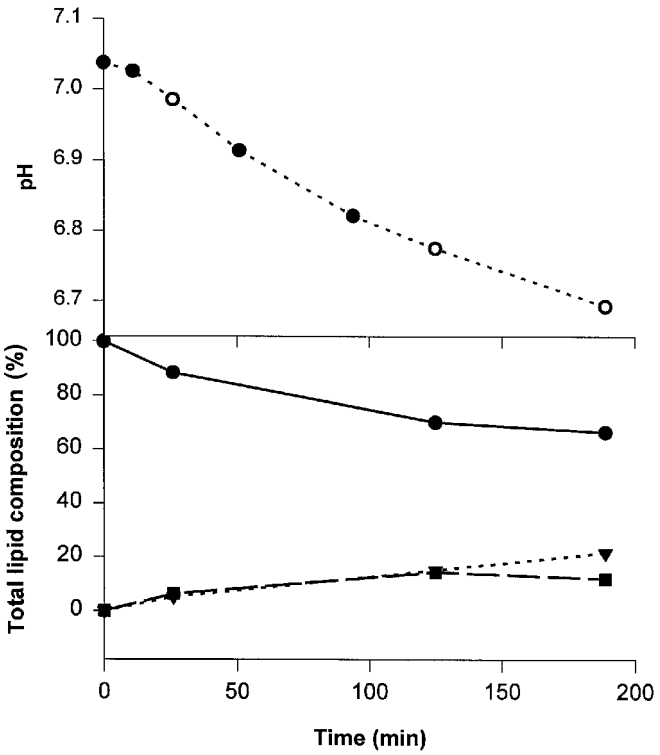


FIG. 2. Example I. LPGL-catalyzed hydrolysis of 19.6 mM TBG, 37°C, 100 mM tris(hydroxymethyl)aminoethane (Tris), 2.5 mg partially purified LPGL. Upper plot: pH vs. time data. The reaction mixtures labeled (○) were sampled for ^{13}C NMR. Lower plot: ^{13}C NMR product analysis with time; 1,2,3-triacylglycerol (TAG) (●), 1,2(2,3)-DAG (■), 1,3-DAG (▼). See Figure 1 for other abbreviations.

At first sight, it might appear that, because the concentrations of 1,2(2,3)- and 1,3-DAG are essentially identical for approximately the first 130 min of incubation, the enzyme hydrolyzes both the 3(1)- and 2-positions, giving rise to the observed pattern of data. However, a close look at the data shows that even in this relatively slow reaction, there is early onset of acyl migration, because the mole fraction percentages at 26 and 125 min for 1,2(2,3)-DAG are 6.3 and 14.3, while for 1,3-DAG they are 5.2 and 15.0, respectively, i.e., there is a crossover in relative abundances of the two DAG.

Previous analysis of the speciation at lower reaction rates had always shown 1,3-DAG to be the predominant DAG species. This experiment was the first in which analysis showed an early predominance of 1,2(2,3)-DAG, raising the question as to whether the initial DAG speciation is masked by acyl transfers. However, an increase in reaction rates is required to probe this, which in turn demands greater amounts of enzyme. Further, the employment of quantitative ^{13}C NMR cannot be made with microgram quantities, and significant masses of reaction products are required. Therefore, the problem was not so much a matter of exact statistical replication, but rather that of having sufficient amounts of enzyme to be able to produce reaction rates and reaction product mass on the required scales, more fully to characterize this behavior.

However, it was also important to establish confidence in the quantitative data, especially as lipids were likely to be extracted from the enzyme fraction that might have contributed to the background spectrum, especially when the crude enzyme was used in subsequent experiments as described below. Many spectra were therefore run that represented worst-case scenarios to confirm that variations in baseline contributions were not a significant factor. Moreover, calibration curves of the ratio of ^{13}C integrated intensity for TAG/[constant 1,2(2,3)-DAG + 1,3-DAG] vs. mass (0–300 mg) of added TAG and of mass ratio (0–4.6) vs. mole ratio (0–7.2) of $C_{4:0}$ TAG/extracted enzyme lipids (as determined by ^{13}C NMR) both gave $r^2 = 0.9998$. We are therefore confident that any apparent differences in amounts of designated species are statistically significant.

The problem was then to separate unequivocally the contributions to the observed mole fractions of individual products over the reaction period, thereby probing details of the reaction sequence. As described below, a sequence of reaction conditions was devised, each new experiment building on the results of the previous one, and each one with larger quantities of enzyme. In this way, we have been able to show that, for example, as the catalyzed reaction rate is increased, the apparent degree of hydrolysis at the *sn*-2 position is progressively reduced.

Example II. In this example, the crude enzyme was used, rather than the partially purified extract used in Example I, to ascertain if the sequence of isomer predominance was affected by the quality of the enzyme. The buffer in this and subsequent examples was also changed from Tris to BTP (pK_a 6.8) to increase the buffering capacity in the pH region of interest. The reactions for both Examples I and II were relatively slow, and Figure 3 shows the results for Example II. This reaction sequence shows clear evidence for a changeover of the predominant DAG species in the system as the reaction progresses. When a second similar hydrolysis reaction was carried out and sequential samples were analyzed, 1,2(2,3)-DAG was the dominant DAG isomer at 81 min, but after 125 min, the situation was reversed and 1,3-DAG was dominant, as it always was in similarly slow reactions conducted in the presence of relatively small amounts of enzyme. Long-sample-time slow reactions were also run for baseline comparison, as well as alkaline glycerolysis, for $C_{6:0}$, $C_{8:0}$, and $C_{10:0}$ TAG. Always, either when extracted into $CHCl_3$, or after rehydrating the extracts and allowing them to stand before reextraction, 1,3-DAG was the predominant DAG.

These reactions confirmed the qualitative similarity in catalytic behavior of the purified and the crude enzyme material. This similarity is important because the high reaction rates that were subsequently employed required the use of progressively greater quantities of LPGL enzyme at levels that prohibited the use of the purified enzyme. This catalytic equivalence of crude LPGL thus permitted its substitution for the limited-available purified enzyme (which had limited availability), to extend the investigation into regimes of high reaction rate.

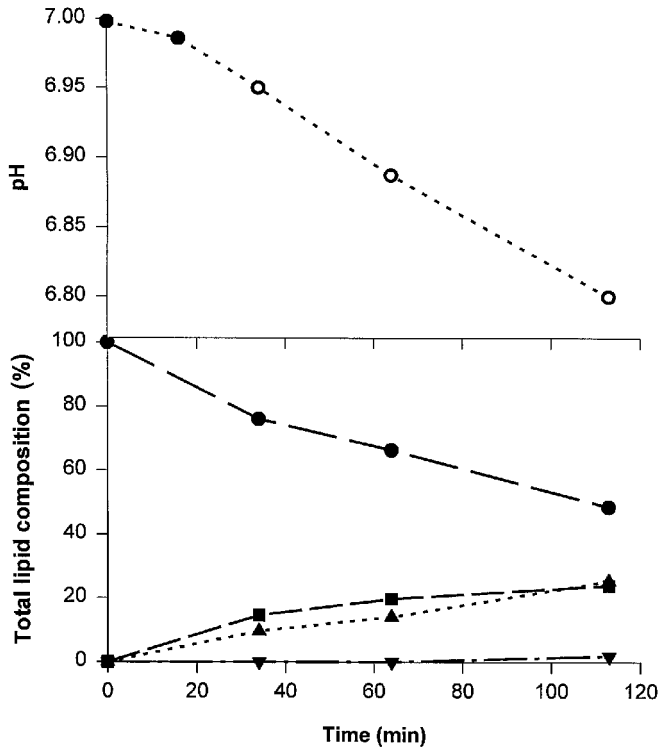


FIG. 3. Example II. LPGL-catalyzed hydrolysis of 28.7 mM TBG, 35°C, 158 mM BTP, 12.5 mg crude LPGL. Upper plot: pH vs. time data. The reaction mixtures labeled as (○) were sampled for ^{13}C NMR. Lower plot: ^{13}C NMR product analysis with time; 1,2,3-TAG (●), 1,2(2,3)-DAG (■), 1,3-DAG (▲), 1(3)-MAG (▼). See Figures 1 and 2 for abbreviations.

Following this initial work, further hydrolyses were performed at increasingly rapid rates to examine the effects of reaction velocity upon the resulting product distribution, and especially the ratios of the 1,2(2,3)-DAG to 1,3-DAG isomers. The following examples detail reactions that were sampled at several time intervals to profile the reaction product sequence, as well as other single-extraction reactions that were intended to produce a large amount of reaction product in a relatively short time. The data derived from these analyses allowed an overview of system behavior and product distribution with variations of reaction rate and elapsed time.

Comparison of the rate effect on resultant product speciation was facilitated by carrying out many reactions under similar conditions of buffering (~200 mM BTP) and of initial pH (pH_i ~7.0). In these experiments. the essential factor was the differing amount of enzyme (i.e., different primary reaction rate). The shift in buffer action with pH change is minor and not affected by small variations (e.g., those in Table 1) in amount of buffer. Furthermore, on the scales used, the reaction is basically zero-order in substrate for the primary hydrolysis but first-order in enzyme. Hence, the only way to increase the reaction rate is to increase the amount of enzyme. Shorter reaction times minimized side reactions, and the necessity to produce quickly sufficient reaction product mass for ^{13}C NMR quantitation demanded the use of large amounts of enzyme. The results confirmed the positional preferences for enzyme-catalyzed hydrolysis of the lipid, and distinguished these species from those arising through other means, such as acyl transfer. Thus, if 1,3-DAG were to arise primarily from acyl transfer conversion of 1,2(2,3)-DAG to 1,3-DAG, we would expect two consequences.

First, for the faster reactions, there should be a higher proportion of 1,2(2,3)-DAG early in the reaction, as well as a delay period before the formation of significant quantities of the 1,3-isomer, because this mechanism for 1,3-DAG production requires the presence of quantities of the 1,2(2,3)-isomer.

Second, we should also be able to demonstrate that 1,2(2,3)-DAG produces 1,3-DAG under similar conditions to those of the hydrolysis but in the absence of any enzymic catalysis. For this latter point to be demonstrated, production of a sample with a high 1,2(2,3)-DAG to 1,3-DAG ratio was required. For this latter point to be demonstrated, production of samples with a high 1,2(2,3)-DAG to 1,3-DAG ratio was required, as in Example VI below. This enzyme-free acyl transfer behavior has been observed in such samples (9).

Example III. The first of the expected behaviors was observed in Example III (Fig. 4), where a substantially higher amount of enzyme was used than in prior experiments, and which exhibits both the high initial predominance of 1,2(2,3)- over 1,3-isomer, as well as an induction period for 1,3-DAG appearance, followed by an increasing rate of 1,3-DAG production. As well as identifying the presence of 1(3)-MAG,

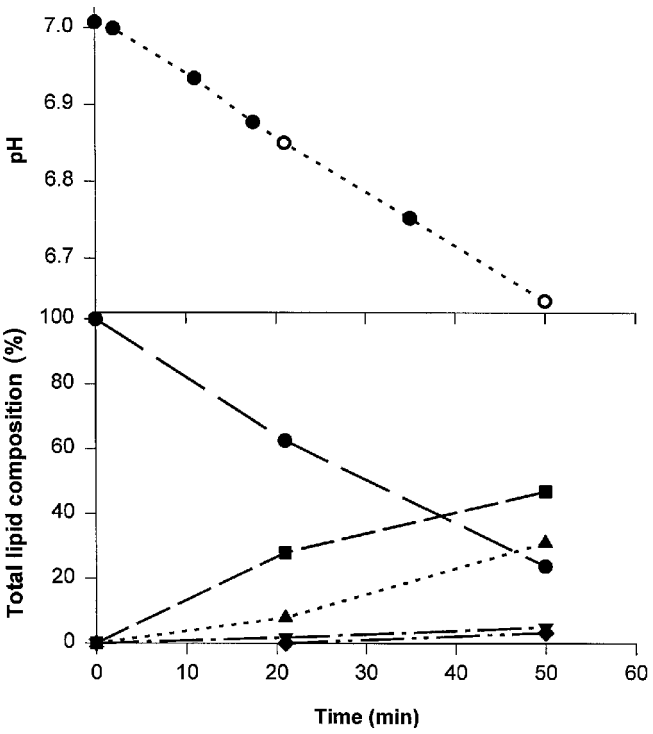


FIG. 4. Example III. LPGL-catalyzed hydrolysis of 34.5 mM TBG, 35°C, 200 mM BTP, 158 mg crude LPGL. Upper plot: pH vs. time data. The reaction mixtures labeled as (○) were sampled for ^{13}C NMR. Lower plot: ^{13}C NMR product analysis with time; 1,2,3-TAG (●), 1,2(2,3)-DAG (■), 1,3-DAG (▲), 1(3)-MAG (▼), 2-MAG (◆). See Figures 1 and 2 for abbreviations.

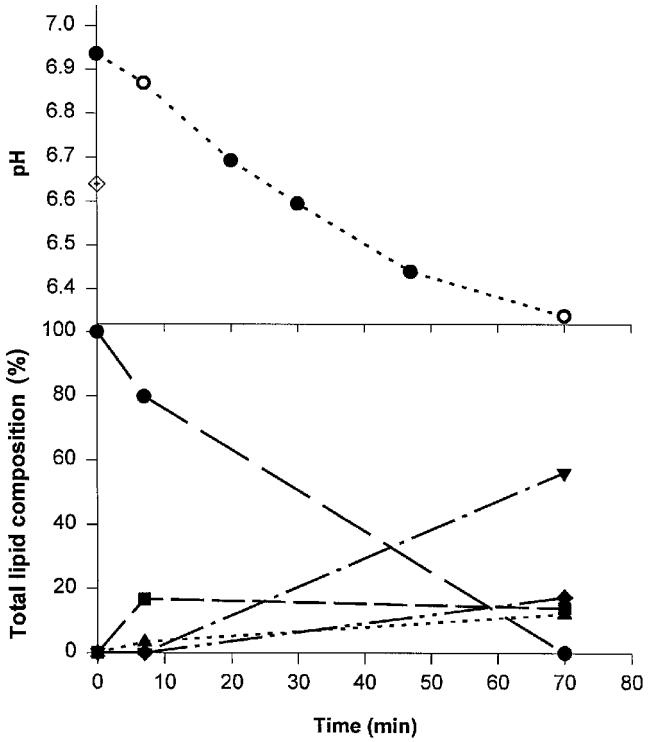


FIG. 5. Example IV. LPGL-catalyzed hydrolysis of 33.9 mM TBG, 35°C, 200 mM BTP, 315 mg crude LPGL. Upper plot: pH vs. time data. The reaction mixtures labeled as (○) were sampled for ^{13}C NMR. Lower plot: ^{13}C NMR product analysis with time; 1,2,3-TAG (●), 1,2(2,3)-DAG (■), 1,3-DAG (▲), 1(3)-MAG (▼), 2-MAG (◆). See Figures 1 and 2 for abbreviations.

which has previously been observed, the first evidence of 2-MAG appeared in this increased-rate reaction. There was also a sharp increase in the fraction of the parent TAG hydrolyzed after 50 min.

Example IV. This example presents an interesting reaction in which rapid hydrolysis was allowed to proceed past complete hydrolysis of the primary TAG substrate. Figure 5 shows the early and late TAG and DAG composition of the mixture. Although the extraction of MAG may not be quantitative, it is believed to represent proportionately the levels of the two MAG present in the reaction mixture. This product distribution suggests an intermediate regime in which the two hydrolysis steps are sufficiently fast to produce a significant quantity of 2-MAG, and where an acyl transfer reaction, which produces 1(3)-MAG, is still relatively fast, thereby allowing it to be the primary MAG present.

Example V (Table 3). This reaction was similar to that described in the Example IV section, but with ~50% more LPGL and approximately twice the reaction time elapsed before removal of the first sample, thereby allowing for greater production of the DAG species, while still retaining some parent TAG, which may inhibit MAG production from the 1,2(2,3)-DAG (see below).

Example VI (Table 3). This example constitutes the highest rate-velocity hydrolysis of TBG that was carried out, and the sample was removed after 15 min of reaction time when the primary TAG substrate was almost completely hydrolyzed.

From these examples, general trends have emerged in the distribution of species, as the reaction velocity is successively increased. These general trends include a progressively greater ratio of 1,2(2,3)- to 1,3-DAG, an increase in the pro-

TABLE 3
Mole Fraction Percentage of Products for LPGL-Catalyzed Hydrolysis of TBG[a]

			Mole fraction (%)				
Example	Time (min)	pH	1,2,3-TAG	1,2(2,3)-DAG	1,3-DAG	1(3)-MAG	2-MAG
V	13	6.70	46.7	41.7	8.44	1.30	1.91
VI	15	6.40	9.4	65.0	11.8	5.7	8.0

[a]Zero time, pH 7.00, 100% 1,2,3-TAG. See Tables 1 and 2 for abbreviations.

portion of 1(3)-MAG, and finally the first appearance of 2-DAG in the extract from more rapid reactions. These features support the conclusion that LPGL is selective for the hydrolysis of primary esters in TAG. While the extraction of MAG species is not believed to be quantitative, the relative proportions in consecutively faster reactions are likely a true reflection of the presence of each species in the extract.

There is evidence that 1(3)-MAG and 2-MAG have the same partition coefficient, and that they have essentially the same polarity, despite the different positions of their primary hydroxyls. By using an internal hydrogen bond between one free hydroxyl and the adjacent carbonyl oxygen, two structures (Fig. 6) can be drawn which are of similar polarity for both MAG, each having only one free hydroxyl group at an end position (19).

Reaction scheme. The equilibrium distribution between the MAG is typically ~90% 1(3)-MAG and 10% 2-MAG (20), with the 2-MAG undergoing relatively rapid acyl transfer to 1(3)-MAG. 2-MAG, the product of two hydrolytic events, is only observed in the more rapid reactions, as opposed to 1(3)-MAG, which appears eventually in extracts from all but the slowest reactions. The increasing presence of 2-MAG as the reaction rate is increased (Fig. 5 vs. Fig. 4) suggests that the production of 2-MAG results from an enzyme-catalyzed hydrolysis of 1,2(2,3)-DAG [because the 2-MAG cannot be formed from 1,3-DAG by any single reaction, and the equilibrium formation of 2-MAG from 1(3)-MAG is highly unfavorable]. The appearance of 1(3)-MAG may then be the result of rapid migration from 2-MAG. If this is true, at sufficiently fast reaction velocity, 2-MAG should predominate over 1(3)-MAG (Example V, Table 3). A reaction scheme summarizing the above, and which is consistent with all these data, is outlined qualitatively in Scheme 1.

Particular features expected to follow from such a reaction scheme depend on the range of predictions concerning the relative rates of the catalyzed and uncatalyzed reactions. The comparative rate constants given are intended to apply only within each reaction class, namely catalyzed hydrolysis (k_1, k_2, k_3) and uncatalyzed acyl transfer (k_4, k_5) reactions. Thus, for small amounts of LPGL, the predominant products should be 1,3-DAG and possibly 1(3)-MAG (if a reasonable amount of 1,3-DAG is produced). In this situation, the uncatalyzed

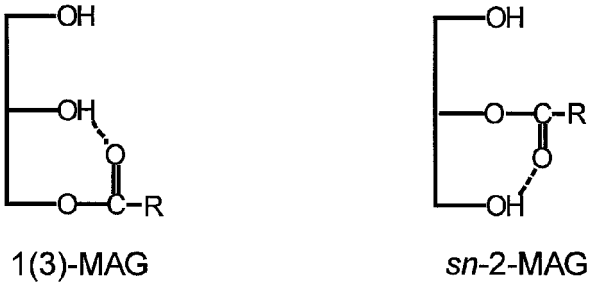
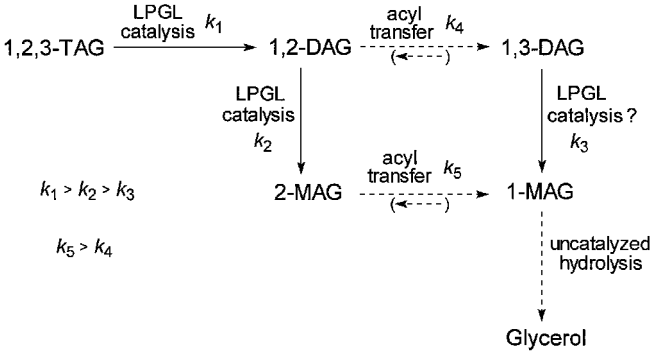


FIG. 6. Structures showing possible internal hydrogen bonding in the two classes of MAG, leading to equivalent polarity (Ref. 14). See Figure 1 for abbreviation.



SCHEME 1

acyl transfer reaction (k_4) will not be rate-determining. As the LPGL-catalyzed rates become faster, 1,2(2,3)-DAG should start to predominate over 1,3-DAG, though 1(3)-MAG will still be primarily produced by hydrolysis of 1,3-DAG. For this latter step, a slow catalyzed reaction (k_3) seems likely because the acyl transfer study (9) confirmed a slow rate of hydrolysis in the absence of enzyme under otherwise similar conditions. However, k_3 may be affected by a nonspecific catalyzed reaction.

As the LPGL-catalyzed rate increases still further with increasing concentration of added enzyme, the 2-MAG proportion of total MAG will increase because the proportion of 1,2(2,3)-DAG present in the early stages of the reaction is greater, and the rate of its conversion to 2-MAG is also increased. As this catalyzed pathway becomes sufficiently rapid, 2-MAG will start to predominate over 1(3)-MAG because production of 1(3)-MAG under these conditions will depend on uncatalyzed acyl transfer reactions (k_4 and k_5), and these will be relatively slower than the catalyzed hydrolysis reactions (k_1 and k_2). The conditions of 35°C and pH 7.0 are nearly optimal for hydrolysis of TBG (12). From our observations during these and other studies (9), we can estimate the value of the acyl transfer rate constant k_4 to be ~0.25 h^{-1} (35°C, pH = 7). The value of k_1, unlike k_4, will depend on the quantity of enzyme employed as catalyst and may thus range from zero to values far greater than k_4. In Example VI, where TAG is largely removed within 1 h, the value of k_1 will be greater than 2.0 h^{-1}). It seems from the above results that the ratio of 1,2(2,3)-DAG to 1,3-DAG will increase with increasing rates of catalyzed reaction.

We have previously shown (12) that the relative rates of hydrolysis for TAG/total DAG were ~25:1, confirming that $k_1 > k_2$. The work of Richardson and Nelson (21) indicated some competitive inhibition of TAG hydrolysis by di- and monobutyrylglycerols and that the Michaelis constant, K_M, increased in their presence. In these present studies, in which the substrate concentration [S] >> K_M, the effect of DAG on the rate of TAG hydrolysis is minimal.

Example IV presents an interesting case in which a rapid hydrolysis reaction was allowed to proceed past complete hydrolysis of the primary TAG substrate, and provides further detail relevant to the mechanisms shown in Scheme 1. Figure

5 shows the reaction pH vs. time profile and the early and late TAG and DAG composition of the mixture. Although the extraction of MAG may not be quantitative, it is believed to represent the proportional levels of the two MAG present in the reaction mixture.

The product distribution observed for Example IV suggests an intermediate regime in which the first two steps are sufficiently fast to produce a significant quantity of 2-MAG, and where the acyl transfer reaction that produces the 1(3)-MAG is still relatively fast, allowing 1(3)-MAG to be the predominant MAG present.

Unfortunately, in this protocol, the long period that elapsed before the last sample was collected may have allowed sufficient time for significant amounts of 2-MAG to be converted to 1(3)-MAG after the primary production of 2-MAG had ceased. The initial rate of conversion of TAG indicates that it should have disappeared well before the last sample was removed, as confirmed by the experiment. The pH for monohydrolysis of the TAG (marked on the y axis in Fig. 5, upper plot) occurs at *ca.* 22 min, suggesting the early disappearance of TAG, though other hydrolysis reactions may have made small contributions to the ΔpH observed at this time, and hence, residual TAG may still be present at this pH.

Variation of catalyzed reaction rate. As a result of these experiments, it is now possible to comment on the relationship of the potential mechanisms implicit in Scheme 1, and the interaction of reaction rates with observed composition of the system, so that the variety of examples and complex features observed for these reactions may be assessed within the overall context of the reaction scheme.

It is known that, in the presence of small quantities of enzyme, the relative rates of hydrolysis of TAG to hydrolysis of the total DAG present are ~25:1, as evidenced both by the rate ratios within the stoichiometry runs (12) before and after complete hydrolysis of TAG, and also from the data of Richardson and Nelson (21). However, the observed profiles of pH vs. time (Figs. 4,5) suggest that this ratio does not hold in the presence of larger amounts of enzyme. If one looks particularly at Figure 5, although the rate of hydrolysis is largely sustained as shown by the falling pH, all TAG is exhausted by 70 min and possibly even a little before this time. Yet, by this time, 56% of the product is present as 1(3)-MAG, indicating that the rate of hydrolysis of DAG is much greater than 1/25th of the rate of hydrolysis of TAG. This increase in relative rates suggests that, in the presence of large quantities of enzyme, there is a switching of the predominant pathway leading to production of the MAG species.

For a low level of enzyme, the suggested rate-determining step is the hydrolysis of TAG (k_1), and the predominant pathway for the reaction is TAG → 1,2(2,3)-DAG → 1,3-DAG → 1(3)-MAG. When MAG was observed in such slow reactions, it was invariably almost exclusively 1(3)-MAG. However, as the amount of enzyme becomes relatively large, the predominant pathway becomes TAG → 1,2(2,3)-DAG → 2-MAG → 1(3)-MAG, with a progressive decrease in rate for each successive step. When the first two steps are sufficiently fast, production of 2-MAG will dominate the MAG species present, provided that the sampling occurs before the MAG have sufficient time to move toward their equilibrium proportions. Production of 2-MAG may be further enhanced by removal of competitive inhibition of the 1,2(2,3)-DAG hydrolysis by any remaining TAG, should this be occurring. Some evidence of the converse, with DAG competitively inhibiting the hydrolysis of TAG, has been briefly noted in the literature (21). In such a fast LPGL-catalyzed reaction, not only is there little time for the 1,2(2,3)-DAG → 1,3-DAG migration but the TAG concentration is also quickly reduced. Therefore, if there were some competitive inhibition of DAG hydrolysis by TAG, the reduction of TAG to low levels by fast reaction [before much 1,2(2,3)-DAG is converted to 1,3-DAG] should allow a further increase in the relative rate of the 1,2(2,3)-DAG → 2-MAG catalyzed reaction.

It should be feasible to resolve an increased proportion of 2-MAG in an extremely fast reaction where the products are sampled both before and near to the complete removal of TAG from the reaction mixture. Early in the reaction, the primary products will be 1,2(2,3)-DAG and 1,3-DAG, with 1,2(2,3)-DAG predominant, due to the rapid rate of its production from catalyzed hydrolysis of TAG. If this extremely high concentration of enzyme then continues to react upon the relatively large quantity of 1,2(2,3)-DAG, which is formed as the concentration of TAG becomes small, the products should show an increase in the ratio of 2-MAG to 1(3)-MAG, to the point where 2-MAG predominates. Such a predominance is rarely observed.

In slow hydrolyses, once all TAG has reacted, the presence of significant 1,3-DAG may also competitively inhibit hydrolysis of the 1,2(2,3)-DAG and hence produce the relatively slow rates observed for hydrolysis of the total DAG present, even in the absence of TAG. These considerations allow the mechanistic pathways outlined in Scheme 1 to cover qualitatively the range of behaviors observed for these reactions.

In summary, the change in the predominant reaction pathway as the enzyme concentration is changed from low to high may be confirmed by increasing the LPGL level still further and sampling at an appropriate time. An extremely high concentration of enzyme should ensure that the acyl transfer step (k_5) becomes rate-determining in production of 1(3)-MAG. Examination of Scheme 1 predicts that, for a sample taken from such a fast reaction soon after the TAG has been eliminated, the predominant MAG species should be 2-MAG (Example VI). Thus, both the rate of reaction and the sampling time will be important to observation of the resultant composition.

The speciation data (Table 3) show two interesting features which were to be expected for catalyzed reactions with high rates, namely that 1,2(2,3)-DAG predominated, comprising ~85% of the DAG present, and that more 2-MAG was present than 1(3)-MAG. The concentration of residual TAG was small, as was that for each of the MAG species.

It is difficult to assess the fate of these MAG, but the speciation observed, together with the 1(3)-MAG to 2-MAG ratios obtained from extractions of other hydrolysis reactions, quali-

tatively suggested that 2-MAG was converted to 1(3)-MAG and that the MAG present also underwent some hydrolysis. These processes were thought to be a significant contributing cause of this decrease in the concentration of MAG, rather than the loss simply being due to poor recovery of MAG.

*The parameter ΔpH/*Δt. Among the trends emerging from consideration of the above examples is the apparent predominance of 1,2(2,3)-DAG as the primary product of the LPGL-catalyzed hydrolysis of TAG, together with an increase in the proportion of 1,3-DAG as the reaction proceeded. This trend has not previously been quantified. Analysis of the ratios of these isomers as a function of reaction rate and reaction time is desirable as an indicator of the effect of reaction rate on the speciation of DAG produced as the primary products of LPGL-catalyzed hydrolysis. To outline this relationship, a method of representation of the reaction rate was required. In view of the parallel and complex interaction of reactions and reaction rates occurring, and the incomplete information available on some of these aspects, a simple generalized reaction rate parameter, $\Delta pH/\Delta t$, was chosen. It is defined as the change in pH (ΔpH) from pH_i to the pH at the time of the sample being removed, divided by the time elapsed (Δt) before removal of that sample. Because the pK_a of butyric acid is 4.82, at the pH values typical of these reactions, the butyric acid produced will be almost completely dissociated (typically >99%), with the consequent release of protons. Because each hydrolysis reaction releases butyric acid and thus yields a proton, the ΔpH in a buffer of known volume, concentration, and pK_a is a measure of the net amount of hydrolysis that has occurred.

The value of $\Delta pH/\Delta t$ is a crude estimate of the reaction rate, in that it is derived from many differing spans of time and of ratios of enzyme to substrate, and is always referred to the commencement of the experiment when only TAG is present, and not to the composition of the previous sample. The early contribution to the value of this parameter is the result of the hydrolysis of TAG to form DAG, and for a known pH_i value, the reaction rate may be precisely calculated from the relevant pH value at the time of sampling. However, as the other reactions of DAG hydrolysis to MAG and of MAG hydrolysis to glycerol come into play, the change of pH ceases to reflect only the rate of the primary TAG hydrolysis.

As can be seen from the pH vs. time profiles (Examples I–IV), the rate of change of pH was often not a linear function of time. A range of properties for the system, as well as the effects of the differing experimental conditions such as the rate of stirring, the temperature and the pH, has the potential to influence the value of $\Delta pH/\Delta t$.

The choice of BTP buffer (pK_a ~6.8, 25°C) was made because the typical pH values encountered over the course of the reactions tended to straddle the value of pK_a, thus maximizing the linearity of buffering. Over the pH range 7.0–6.6, the effect of a change in pH on enzymic activity (12) was also minimized. Moreover, a neutral pH avoids autolysis of TBG, which does not become significant until pH ~8 (21,22). Thus, pH_i ~7.0 meets requirements to best advantage and avoids other problems, such as general acid- or base-catalyzed hydrolysis.

Nevertheless, the parameter $\Delta pH/\Delta t$ is still a coarse measure of reaction rate and will vary within each reaction run, as can be seen from the nonlinearity observed in many of the pH vs. time plots.

A plot (not shown) of the $\Delta pH/\Delta t$ parameter against the mass of enzyme for Examples I–VI was reasonably linear, despite the effects of a wide range of reaction times and compositions, and this parameter may therefore be used to formalize a feature of the reaction system, which up to now has been only qualitatively characterized. Figure 7 shows a plot of the fraction of total DAG appearing as 1,2(2,3)-DAG as a function of the reaction time, Δt, and the logarithm of the derived parameter for the approximate rate of reaction, $\Delta pH/\Delta t$. All data were taken from runs with pH_i ~7.0, and $\Delta pH/\Delta t$ was corrected to 200 mM^{-1} equivalents of buffer concentration. This plot shows that there is a general increase in the proportion of 1,2(2,3)-DAG as the reaction rate is increased and as reaction time is decreased. This behavior is expected when an enzyme-catalyzed hydrolysis reaction produces primarily 1,2(2,3)-DAG and is accompanied by a nonspecific conversion of 1,2(2,3)-DAG to 1,3-DAG. This latter reaction has been found to occur in the absence of enzyme (9).

This investigation did not lead to a definitive limit on the rate selectivity of the 1,2(2,3)/1,3 positional attack because increase of rate or decrease of reaction time produced a corresponding increase in the proportion of the 1,2(2,3)-DAG component of total DAG. However, it seems likely that Scheme 1 represents a system in which the first catalyzed hydrolysis is entirely *sn*-1(3)-selective, and all 1,3-DAG is produced by (nonspecific) acyl migration from 1,2(2,3)-DAG. In other words, no theoretical restriction is placed on the possi-

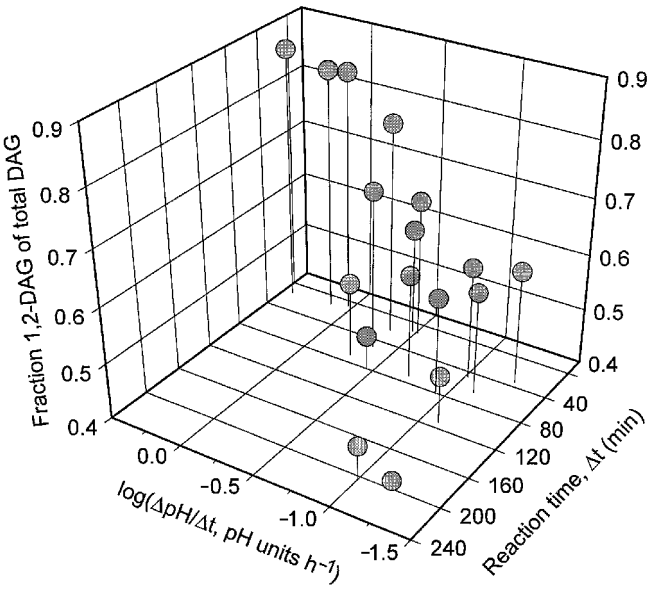


FIG. 7. DAG isomer composition as a function of the logarithm of the average rate of change of pH ($\Delta pH/\Delta t$) and reaction time (Δt) for LPGL-catalyzed hydrolysis of different masses of TBG, pH_i ~ 7.0, 35°C. See Figure 1 for abbreviations.

bility that, for progressively faster reaction rates and increasingly short reaction times, the DAG speciation will tend toward 100% 1,2-/2,3-isomer. However, there are experimental limitations because reactions with high rates are not usually employed due to the demand for large amounts of enzyme, and ultrashort reaction times are not widely investigated because they yield such small amounts of converted material. In this study, for faster reactions with relatively short sample times, DAG isomeric compositions up to and including ~86% 1,2(2,3)-DAG [1,2(2,3)-DAG:1,3-DAG > 6:1] were achieved several times in the most rapid reactions carried out.

A more clearly delineated formal limit was the progressive decrease toward the equilibrium composition of the 1,2-isomer [to ~35–40% 1,2(2,3)-DAG], brought about by acyl transfer under the conditions described. This limit composition for DAG applied only to slow reaction rates and/or long reaction times.

In conclusion, the LPGL-catalyzed hydrolysis of TBG, carried out under a range of reaction times and enzyme concentrations, shows properties of composition and reaction rates that are consistent with the mechanism outlined in Scheme 1. The resolution and separation of the effects of acyl transfer and of catalytic action have allowed the general positional selectivity of LPGL (for inner or outer positions of the TAG) to be specified as a preference for a single outer position. Previous indication of stereopositional preferences has given ratios of reported rates of 4:1 (human) and 2:1 (rat) for *sn*-3 to *sn*-1 attack with the PGL noted (23,24). In particular, speciation of the DAG present in the system depends strongly upon the rate of the catalyzed reaction and the elapsed reaction time and confirms the strong positional selectivity of enzyme-mediated hydrolysis.

ACKNOWLEDGMENTS

Equipment grant from Auckland University Research Committee and New Zealand Lottery Science and a PhD Fellowship (to RHB) from the New Zealand Agricultural and Marketing Research Development Trust are gratefully acknowledged. We are indebted to Dr. Keith Turner and the New Zealand Rennet Company Ltd. for the kind gift of enzyme and continued interest.

REFERENCES

1. Birschbach, P., Pregastric Lipases. *Bull. Int. Dairy Fed. 269:*36–39 (1992).
2. Nelson, J.H., R.G. Jensen, and R.E. Pitas, Pregastric Esterases and Other Oral Lipases, *J. Dairy Sci. 60:*327–362 (1977).
3. Hamosh, M., Gastric and Lingual Lipases, in *Physiology of the Gastrointestinal Tract,* 3rd edn., edited by L.R. Johnson, Raven Press, New York, 1994, p. 1239.
4. O'Connor, C.J., R.H. Barton, P.A.G. Butler, A.D. MacKenzie, R.D. Manuel, and D.T. Lai, Ruminant Pregastric Lipases: Experimental Evidence of their Potential as Industrial Catalysts in Food Technology, *Colloids Surf. B: Biointerfaces 7:*189–205 (1996).
5. Hamosh, M., Lingual Lipases, in *Lipases,* edited by B. Borgström and H.L. Brockman, Elsevier, Amsterdam, 1984, p. 49.
6. Gunstone, F.D., in *The Lipid Handbook,* 2nd edn., edited by J.L. Harwood and F.B. Padley, Chapman & Hall, London, 1994, p. 195.
7. O'Connor, C.J., D.T. Lai, and R.H. Barton, Lamb Pregastric Enzyme-Catalysed Hydrolysis of 4-Nitrophenylalkanoates and Monoacid Triglycerides, *J. Mol. Cat. B: Enzymatic 1:*143–149 (1996).
8. O'Connor, C.J., and R.D. Manuel, Calf Pregastric Lipase Catalysed Hydrolysis of Short- and Medium Chain-Length Monoacid Triglycerides: Temperature, pH and Lipid Concentration Effects, *Prog. Colloid Polym. Sci. 106:*188–191 (1997).
9. Barton, R.H., and C.J. O'Connor, Uncatalysed Intramolecular Acyl Transfer in a Lipid Hydrolysis Product Mixture, *Aust. J. Chem. 50:*355–361 (1997).
10. Tso, P., Gastrointestinal Digestion and Absorption of Lipid, *Adv. Lipid Res. 21:*143–186 (1985).
11. O'Connor, C.J., T.D. Lai, and R.H. Barton, Lipolytic Synthesis of Optically Active 1,2-Dibutyryl-*sn*-glycerol. Identification of Diglyceride by Solvent-Dependent Specific Rotation, *J. Am. Oil Chem. Soc. 75:*1061–1062 (1998).
12. Barton, R.H., C.J. O'Connor, and K.W. Turner, Characteristics of Tributyroylglycerol Hydrolysis Mediated by a Partially Purified Lamb Pregastric Lipase, *J. Dairy Sci. 79:*27–32 (1996).
13. Gunstone, F.D., ^{13}C-NMR Spectra of Some Synthetic Glycerol Esters Alone and as Mixtures, *Chem. Phys. Lipids 56:*195–199 (1990).
14. Lie Ken Jie, M.S.F., C.C. Lam, and B.F.Y. Yan, ^{13}C NMR Studies on Some Synthetic Saturated Glycerol Triesters, *J. Chem. Res. (M):*250–272 (1992).
15. Gunstone, F.D., ^{13}C-NMR Studies of Mono-, Di- and Tri-acylglycerols Leading to Qualitative and Semi-Quantitative Information About Mixtures of These Glycerol Esters, *Chem. Phys. Lipids 58:*219–224 (1991).
16. O'Connor, C.J., S.F. Petricevic, J.M. Coddington, and R.A. Stanley, An NMR Assay for Quantitating Lipase Activity in Biphasic Macroemulsions. *J. Am. Oil Chem. Soc. 69:*295–300 (1992).
17. Folch-Pi, J., M. Lees, and G.H. Sloane Stanley, A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226:*497–509 (1957).
18. Bligh, E.G., and W.J. Dyer, A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37:*911–917 (1959).
19. Privett, O.S., and L.J. Nutter. A New Method for the Analysis of Component Mono-, Di-, and Triglycerides, *J. Lipid Res. 2:*37–44 (1961).
20. Buchnea, D., Synthesis of Enantiomeric Acylglycerols, in Handbook of Lipid Research 1: Fatty Acids and Glycerides, edited by A. Kuksis, Plenum Press, New York, 1978, p. 233.
21. Richardson, G.H., and J.H. Nelson, Assay and Characterisation of Pregastric Esterase, *J. Dairy Sci. 50:*1061–1065 (1967).
22. Castberg, H.B., P. Solberg, and T. Egelrud, Tributyrate as a Substrate for the Determination of Lipase Activity in Milk. *J. Dairy Res. 42:*247–253 (1975).
23. Jensen, R.G., F.A. de Jong, R.M. Clark, L.G. Palmgren, T.H. Kiao, and M. Hamosh, Stereospecificity of Premature Human Infant Lingual Lipase, *Lipids 17:*570–572 (1982).
24. Paltauf, F., F. Esfandi, and A. Holasek, Stereospecificity of Lipases. Enzymic Hydrolysis of Enantiomeric Alkyl Diacylglycerols by Lipoprotein Lipase, Lingual Lipase and Pancreatic Lipase, *FEBS Lett. 40:*119–123 (1974).

[Received August 5, 1997; accepted March 4, 1998]