An NMR Assay for Quantitating Lipase Activity in Biphasic Macroemulsions

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A novel nuclear magnetic resonance (NMR) assay has been developed to monitor lipase-catalyzed esterification reactions without the need to extract and purify the individual components. The technique measures ratios of ester:alcohol signals and has been shown to be both efficient and reproducible. The assay has proven useful as a quick screen for the effect of varying conditions on the extent of esterification in various biphasic solvent systems and can be applied to both saturated and unsaturated long-chain fatty acid and alcohol substrates. The NMR ratio technique has been used to quantitate the extent of reaction in the *Candida cylindracea* lipase-catalyzed synthesis of oleyl palmitate, stearyl palmitate, oleyl oleate, oleyl *gamma*-linolenate and oleyl linoleate. The identity of these products was confirmed by high-resolution mass spectrometry.

KEY WORDS: *Candida cylindracea*, esterification, ester synthesis, lipase activity, macroemulsions, NMR assay, positional specificity of enzyme reactions.

We have previously reported that *Candida cylindracea* lipase catalyzed the hydrolysis of methyl palmitate in paraffin/water biphasic macroemulsions (1). However, the results indicated that the reaction equilibrium lay toward formation of the ester. We have recently investigated the reverse reaction (2) and found that, at equilibrium, *ca.* 75% esterification had occurred between palmitic acid and oleyl alcohol. Analysis in both these investigations was by titrimetric assay of the free fatty acid after converting it to the sodium salt, extracting it into an equivolume mixture of ethanol/water and back-titrating it with acid.

The main difficulty with this titrimetric technique to analyze the esterification reactions was that a rapid change in the water content occurred during the reaction. The overall catalytic process is regulated by a two-dimensional surface phase, which separates the lipid droplet from the aqueous milieu (3,4), and the surface concentrations are themselves determined through partitioning reactions between the bulk phases and the surface phase. The reactions under investigation are often efficient and result in the formation of large amounts of water and ester products. In turn, these agents will influence the stability of the emulsion particles, which may undergo coalescence and exhibit size heterogeneity. We concluded that the titrimetric assay, which depended initially on solvent extraction of fatty acid from a non-homogeneous mixture of constantly changing composition, was suitable for use as an indicator of the extent of esterification, but less so for quantitative analysis.

Lipases are of growing interest as catalysts in fat technology (5,6) and in organic synthesis (7,8), but our understanding of the mechanism of lipolysis and its optimization remains limited (4,9). One reason for this may be that, although the activity of lipases strongly depends on the physical state of the substrate, *i.e.*, on the way the lipophilic substrate is presented to the enzyme (4), there is no one satisfactory analytical technique available for monitoring the course of reactivity of a wide variety of substrates in a range of solvents.

We outline below a novel nuclear magnetic resonance (NMR) technique that does not measure the amount of free fatty acid released but rather the ratio of ester:alcohol in the reactant mixture. Therefore, it may be used with relatively crude substrates and for the high concentrations that are needed for biocatalytic ester synthesis for industrial purposes. We have obtained satisfactory analyses for macroemulsion media containing 100 mM of free fatty acid. The upper limit of this technique is dictated only by the solubility of the substrates in the organic phase of the emulsion. The analysis may be used for a wide variety of solvent mixtures and does not require extraction of products or reactants prior to analysis. We believe that this efficient NMR method has wide applications for monitoring esterification reactions in biphasic organic/water solvent systems.

MATERIALS AND METHODS

Reagents. The enzyme *Candida cylindracea* lipase was type VII from Sigma Chemical Co., St. Louis, MO, and was used as obtained. Cyclohexane (Riedel chromatographic grade), hexane and *iso*-octane (Shell reagent-grade), and paraffin [Shell oil 15 (medicinal)] were used as obtained. Oleyl alcohol and palmitic acid were from BDH (Poole, Dorset, United Kingdom), oleic acid from M&B (Dagenham, Essex, United Kingdom), stearyl alcohol was Riedel-de Haën (Seelze, Germany) analytical reagent, and *gamma*-linolenic acid, 77% pure, was a kind gift from Scotia Pharmaceuticals, Guildford, United Kingdom. Oleyl palmitate, stearyl palmitate, and *cis*-linoleic acid (all 99% pure) and linolenic acid (98% pure) were from Sigma.

NMR spectroscopy. ^{13}C NMR spectra were recorded at 100 MHz in a Bruker (Bodensee, Germany) AM 400 spectrometer equipped with a Bruker 5-mm ^{1}H-^{13}C dual probe. Spectra were acquired with a 70° pulse (4.0 μsec) and 10.0 sec relaxation time to allow complete relaxation of all signals, including the carbonyl carbons. Composite pulse broadband decoupling was used during acquisition.

The free induction decay (FID) spectra obtained were processed with a line broadening of 2.5 Hz, phase corrected and Fourier transformed to yield frequency domain data. Spectra were printed out in the region of 200 to −10 ppm and referenced to tetramethylsilane (TMS).

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The regions used for both quantitative and qualitative work were those between 172–182 ppm and 62–66 ppm. The first region was monitored for the appearance of a quaternary carbon signal at *ca.* 174 ppm, indicative of the presence of an ester carbonyl linkage $-CH_2-O-CO-$. (The acid carbonyl signal was much smaller than that of the ester and would have required a ten-fold greater investment in collection time to allow the same confidence level in assignment.) The second region initially showed the alcohol $-CH_2-OH$ secondary carbon at *ca.* 63 ppm. The emergence of a secondary carbon signal at *ca.* 64 ppm attributable to the $-CH_2-O-CO-$ linkage of the ester product could be observed. The multiplicities of the ^{13}C signals were assigned by performing 90° and 135° DEPT editing experiments. The signals in region two were integrated by using the algorithm in the manufacturer-supplied software and the ratio of 63:64 (ester:alcohol) signal area recorded. The reproducibility of the method was examined by analyzing a standard mixture of oleyl palmitate and oleyl alcohol (equivalent to 75% esterification). The mixture was analyzed ten times by the method described above (but with a reduction in the number of scans from 64 to 16 in order to conserve instrument time). In each case, a Fourier transformation was performed on the FIDs under the same conditions, and acquisition parameters were kept constant. The start and end points of the ester and alcohol signal integrals were chosen manually, and the ratio of ester:alcohol was determined. The mean ratio was 0.187 with a standard deviation of 0.010 (5.4%). Experimental results obtained with the increased number of scans (which were used for the lipase assays) would be expected to give a lower standard deviation, due to approximately doubled signal:noise ratio observed with a quadrupling in the number of scans.

Preparation of a calibration curve. Mixed standard solutions of oleyl alcohol and oleyl palmitate corresponding to the range of concentrations present between 0% to 100% esterification in the biphasic system described below (*ca.* 4.3 equivalents of alcohol to acid) were prepared. The maximum number of moles of alcohol used was 5.33×10^{-4}, decreasing by the number of moles of ester produced to a minimum of 4.11×10^{-4} moles. Each standard was diluted with 0.5 mL of $CDCl_3$ containing 0.03% TMS and analyzed by ^{13}C NMR as described above.

Nuclear Overhauser Effect. The intensity of decoupled ^{13}C NMR signals, such as those used for this work, is known to be enhanced by the collapse of coupled proton multiplets and is also affected by the Nuclear Overhauser Effect (NOE). This effect is observed when the population distribution of carbon energy levels is disturbed by proton decoupling, leading to an increased probability of excitations and, therefore, increased intensity of the observed carbon NMR signals. The NOE is dependent on the relaxation behavior of the carbon atom involved and differs with the chemical and physical environment of carbon atoms in a molecule.

The experimental ratio of ester:alcohol signal used could have been influenced by variable NOEs and the alcohol $-CH_2-OH-$ carbon signal could have had different relaxation behavior from the ester $-CH_2-O-CO-$ signal, rendering the integral ratio unreliable. To test this possibility, an experiment was conducted to examine the change in signal intensity observed for each carbon atom in the presence and absence of NOE. A mixed sample of oleyl alcohol and oleyl palmitate recovered from a biphasic macroemulsion synthesis of oleyl palmitate in a 20% *iso*-octane/80% water (v/v) solvent system was prepared, as described above, with five equivalents of alcohol to acid. A sample was removed and centrifuged to separate the organic phase, which was then diluted with $CDCl_3$. Spectra were acquired under normal broadband decoupling conditions and then under inverse gated conditions where no NOEs were allowed to build up.

Preparation of reaction mixtures. The macroemulsion media were prepared as follows. Water (x-mL), corresponding to a 20% v/v fraction (or 5% v/v fraction for the hexane/water solvent system) of 40 mL, was pipetted into a jacketed reaction vessel (internal diameter 55 mm, total volume 150 mL), which was maintained at 37°C. Solvent (*iso*-octane, cyclohexane, paraffin or hexane), (40-x) mL, was added and the resultant biphasic mixture stirred mechanically to form an emulsion. The required quantity of fatty acid and alcohol was dissolved in the mixture. *Candida cylindracea* lipase (0.25 g) was sonicated in 3 mL of water for 5 min to form a suspension of insoluble protein and, at zero time, the suspension was added to the reaction vessel with 2 mL of rinse water. All reactions were carried out with nitrogen ebullition and continuous stirring.

Aliquots (1 mL) were withdrawn at time intervals up to 1440 min, centrifuged at *ca.* 750 *g* for 5 min, and 200 μL of the upper organic layer was then diluted with 0.5 mL of $CDCl_3$ containing 0.03% TMS, filtered into a 5-mm NMR tube, and the carbon-13 NMR spectrum was determined as described above. The distinct advantage of this analytical method was that it could be carried out on the crude reaction mixture without purification of the individual components. Accumulation of the alcohol and ester signals required less than 5 min of instrument time. The experiment was repeated in 20% *iso*-octane/80% water (v/v) solvent system under identical conditions to those described above but for reaction of oleyl alcohol in the absence of palmitic acid, and *vice versa.* The NMR spectrum was measured on a sample removed after 1020 min. In this solvent system the reaction was also run for 180 min in the presence of oleyl palmitate alone. The effect of adding a further 0.25 g of *C. cylindracea* lipase, after a reaction time of 180 min of reaction in this solvent system, was investigated. Samples were removed for analysis after a further 180 min of reaction time.

Purification of products. The crude reaction mixtures examined above were subjected to solvent extraction and column chromatography. In each case, the *iso*-octane layer containing ester product was separated from the reaction mixture, washed twice with methanol:water (93:7) solvent, dried (anhydrous sodium sulfate), filtered and evaporated to dryness. The resultant oil (*ca.* 200 μL) was loaded onto a disposable C18 column, eluted first with 1.5 mL of methanol:water solvent to remove excess alcohol, then with 1.5 mL of *n*-hexane. The hexane fraction was evaporated to dryness to yield an oil, the column chromatography was repeated, and the final *n*-hexane layer was evaporated to dryness. The resultant oil was subjected to NMR and mass spectrometric analysis. Wherever possible, NMR spectra were compared with those of authentic samples. The synthetic samples of oleyl palmitate, stearyl palmitate, oleyl oleate, oleyl *gamma*-linolenate and oleyl linoleate showed no evidence for unreacted alcohol in the purified product.

Mass spectrometric characterization. Low- and subsequent high-resolution mass spectra (recorded at nominal resolution of 3,000 and 10,000, respectively) were run on a VG-70SE (Manchester, England) mass spectrometer. The accelerating voltage was 70 eV, and the instrument was run in the Direct Electron Impactionization mode. This mode was preferred for enhancement of the relatively small molecular ions expected for the hydrocarbon-like long-chain fatty acid esters being examined. Perfluorokerosene was used as an internal standard for each determination.

RESULTS AND DISCUSSION

Assay calibration. An example of a ^{13}C spectrum obtained from a sample mixture of oleyl alcohol, palmitic acid and oleyl palmitate extracted from a 20% *iso*-octane/80% water (v/v) solvent system is shown in Figure 1. Figure 2 shows an example of the change in ester (ppm) to alcohol (ppm) signals with time in the ^{13}C spectrum of a sample mixture of oleyl alcohol, palmitic acid and oleyl palmitate extracted from a 20% *iso*-octane/80% water (v/v) solvent system. A plot of the ester:alcohol NMR signal ratio against the ester:alcohol mole ratio for zero time is shown in Figure 3.

Oleyl palmitate synthesis. The results for the *C. cylindracea* lipase-catalyzed synthesis of oleyl palmitate from 5×10^{-3} mol palmitic acid and 2×10^{-2} mol oleyl alcohol, determined in 20% *iso*-octane, 20% cyclohexane, 20% paraffin or 95% hexane in water biphasic macroemulsions, are shown in Figure 4. The results obtained in 20% *iso*-octane/80% water (v/v) solvent system but using only 1×10^{-2} mol oleyl alcohol (mole ratio acid:alcohol = 1:2) are given in Figure 5. The ^{13}C spectrum of oleyl alcohol, palmitic acid and oleyl palmitate, removed from the macroemulsion medium after exposure of each alone to *C. cylindracea* lipase, was unchanged from that of the corresponding unreacted sample. The addition of fresh *C. cylindracea* lipase to the reaction mixture after 180 min (following equilibration) had no effect on the position of the plateau shown in Figure 4, indicating that equilibrium had been obtained.

The high-resolution mass spectrum of the purified oily product gave a molecular ion *m/z* 506, with an accurate mass of *m/z* 506.50595 (error 0.7 ppm from the calculated formula $C_{34}H_{36}O_2$, oleyl palmitate). A mixture of the characteristic C_nH_{2n} alkene and C_nH_{2n+2} alkane fragmentation patterns was observed in the mass region below *m/z* 200 as expected for the oleyl and palmitate groups, respectively.

The NMR assay. A novel NMR assay has been developed for use as a quantitative tool in monitoring esterification reactions of long-chain fatty acids with alcohols. The technique has been shown to be quick, easy and reproducible. Furthermore, considerable time may be saved over conventional analyses in that the reaction can be monitored without the need for purification of the target compounds. Crude *C. cylindracea* lipase (supplied extended with lactose) has been used in all the synthetic work described because it is of industrial importance. Extended dialysis of this commercial product followed by lyophilization gave a partially purified enzyme (26% of recovered weight). After use in the macroemulsion medium of 20% *iso*-octane/80% water (v/v) solvent mixture, the enzyme had not lost activity, thus indicating the viability of using this lipase in biologically mediated industrial processes. In the synthesis of oleyl palmitate, the NMR assay has

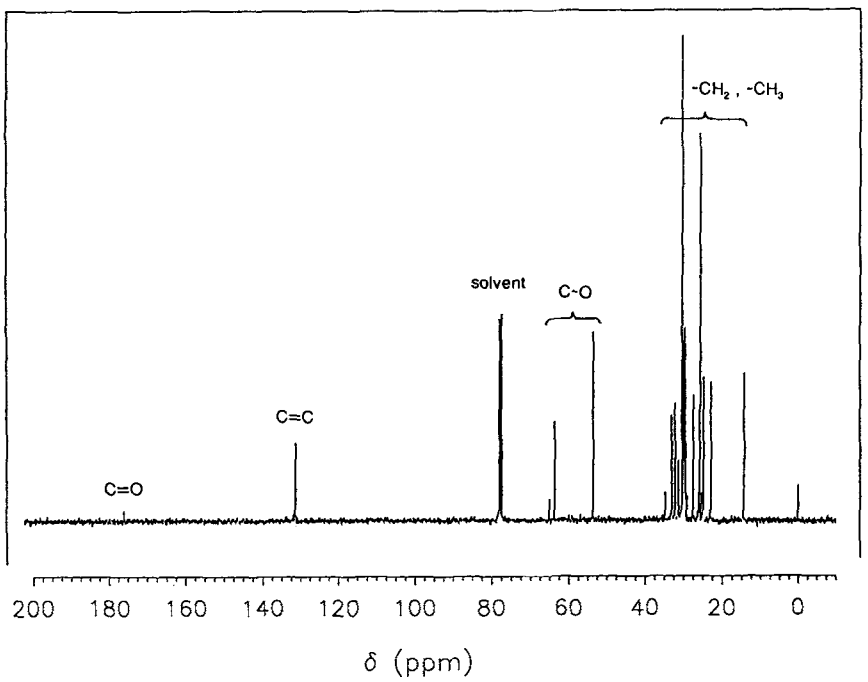

FIG. 1. ^{13}C spectrum of a sample mixture of oleyl alcohol, palmitic acid and oleyl palmitate extracted from a 20% *iso*-octane/80% water (v/v) solvent system.

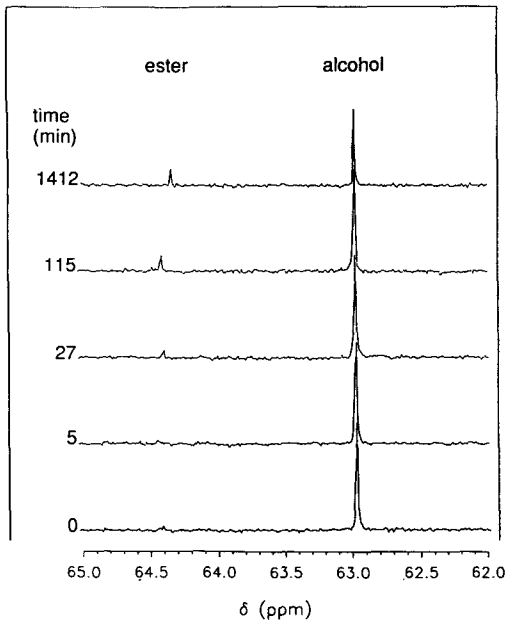

FIG. 2. Increase in the ^{13}C ester signal (64.3 ppm) relative to the alcohol signal with time. Data from oleyl palmitate synthesis in 20% *iso*-octane/80% water (v/v) solvent.

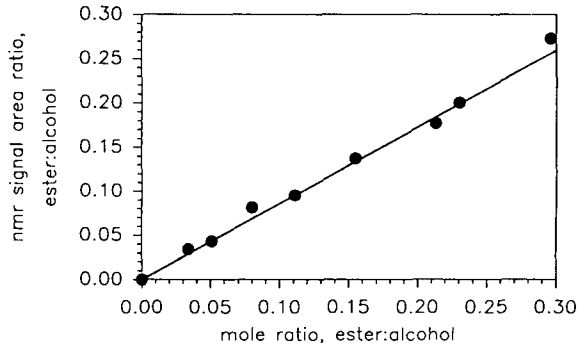

FIG. 3. Calibration curve for *C. cylindracea* lipase-catalyzed synthesis of oleyl palmitate; ^{13}C NMR determination.

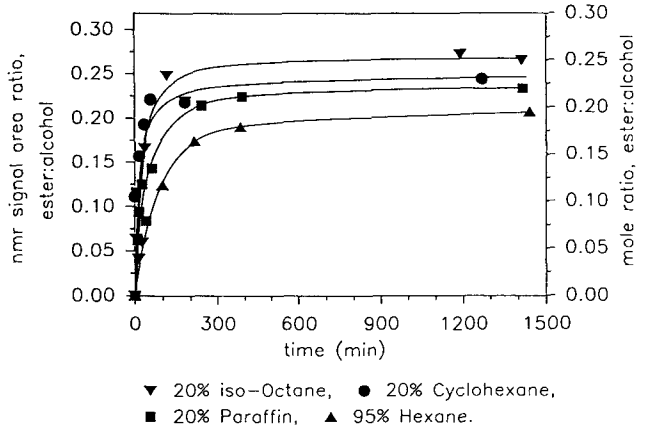

FIG. 4. NMR study of oleyl palmitate synthesis showing the effect of varying the solvent in organic/aqueous (v/v) solvent systems at 37°C. ▼, 20% *iso*-octane/80% water; ●, 20% cyclohexane/80% water; ■, 20% paraffin/80% water; ▲, 95% hexane/80% water. (Initial ratio of acid:alcohol = 1:4.3.)

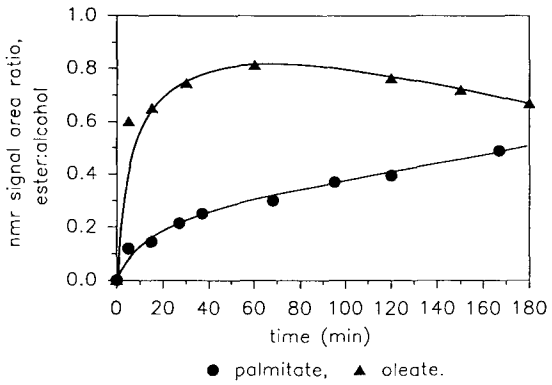

FIG. 5. Ester synthesis in 20% *iso*-octane/80% water (v/v) solvent system at 37°C; ^{13}C NMR determination. ●, oleyl palmitate; ▲, oleyl oleate syntheses at two equivalents of oleyl alcohol. (Initial ratio of acid:alcohol = 1:2.)

proven to be useful as a method of quickly screening the effect of changing a parameter (such as solvent composition) on the extent of esterification. A rapid indication of the applicability of lipases for the synthesis of a variety of saturated and unsaturated ester products can be obtained.

Care has to be taken when using integrated ^{13}C NMR signals for quantitative work. The Fourier-transform conditions applied to FIDs need to be identical if spectra are to be compared in this manner. ^{13}C T1 relaxations have to be considered, and a long enough delay between pulses needs to be applied to ensure that each carbon has relaxed before application of the next pulse. For the current work, a relaxation delay of 10 sec was found to be adequate. In the trial case of synthesis of oleyl palmitate, reproducible results were obtained, and it was shown that the NOE experienced by both the alcohol CH_2-OH and ester -CH_2-O-CO- signals was virtually identical. This meant that the integration performed was valid and that the spectrum digitization was a true reflection of the band shape (10). The integrated ratios of carbon signals obtained both in the presence and absence of NOE were found to be in agreement. Although, as expected, the integrals obtained when NOE was allowed to build up were larger than those collected in the absence of NOE, the signal ratios for the two sets of results concurred. The maximum theoretical NOE enhancement possible for a proton decoupled ^{13}C signal is a factor of 2.988 (10). The results obtained with the NMR quantitative method developed here showed enhancement factors of 2.3 and 2.4 for the ester and alcohol signal, respectively. These results indicate that the NOE for both signals has similar population and relaxation effects, and thus the integration of these signals is valid under standard conditions.

The NMR ratio technique has been developed to quantitate the extent of reaction occurring in enzyme-

catalyzed esterification reactions in macroemulsion media. Specifically, the synthesis of oleyl palmitate, as shown in Figure 4, shows the relationship between the NMR ester: alcohol signals and the mole ester:alcohol ratio. The relationship was linear according to the calibration curve shown in Figure 3. Calibration curves must be established for each system under investigation, and with the appropriate excess of alcohol over ester. The ester and alcohol signals chosen for the ratio measurements were not the only ones that changed during the course of the reactions. They were, however, the most convenient signals.

Samples from emulsions may be readily assayed after only a simple centrifugation followed by dilution. Because the technique effectively uses an internal standard by comparing the ratio of the product to reactant, it is not necessary that each sample assayed be of exactly equal volume. Internal standard added after phase separation would not detect desorption into the aqueous layer arising from formation of surfactant micelles.

One disadvantage of the NMR ratio technique is that an NMR spectrometer may not be available to industry. In such a situation, the method of O'Connor *et al.* (1,2) may be more suited for an industrial in-flow type process that requires only qualitative control. Nevertheless, this new technique could well be used in research and development into specific aspects of enzyme-catalyzed esterifications involving long-chain fatty acids and alcohols in nonaqueous solvents. Reaction conditions could be varied and the process could be monitored on a small scale in the research laboratory before applying the findings to improvement of a known process.

Solvent preferences. The data in Figure 4 for the esterification of oleyl palmitate show a preference in product yield for the 20% *iso*-octane/80% water (v/v) solvent system, and this solvent was used in the other investigations described below. A slight decrease in the extent of reaction was found in 20% cyclohexane or 20% paraffin solvents, but a change to 95% hexane/5% water dropped this value by 25 percent. The lipase must form an equilibrium from the balance of the forward and reverse reactions, and in these systems the equilibrium is heavily in favor of synthesis. Essentially, the organic solvent is inert and dilutes the concentration of reactants, both fatty acid and fatty alcohol. As the fatty alcohol was in excess, we suggest that the efficiency of these systems is due to the binding of the fatty acid to the lipid surface where the enzyme was adsorbed, thus raising the local concentration of the reactant. The ester product on the other hand will be more soluble in the interior of the lipid droplets and is thus of much lower apparent concentration to the enzyme. It is the concentration of reactants at the surface near the active site of the enzyme that will be the dominant effect on the equilibrium of the ester synthesis and not the water concentration. In the shorter-chain n-alkane system, partitioning of substrates and product will be less well differentiated and enzyme efficiency will be inhibited. A further advantage of the 20% organic/80% water (v/v) solvent system is that it gives a high concentration of product and reactants in a smaller volume of organic solvent, thereby enhancing the substrate:solvent ratio. This condition facilitates the acquisition of strong ^{13}C NMR spectra while using a shorter time for the analysis.

Varying the concentration of substrates. A decrease in the ratio of oleyl alcohol:palmitic acid from 4.3:1 (Fig. 4 and ref. 2) to 2:1 (Fig. 5) slowed the rate of net esterification and allowed more accurate determination of the composition of the reaction mixture with time. The quantitated ^{13}C NMR signals of the ester and alcohol were of similar size, one of the requirements for an effective internal standard. Moreover, the decrease in concentration of excess alcohol enhanced the recovery of ester product. There was less emulsification to be broken by centrifugation, the two layers separated more easily, and fewer solvent-wash steps were required in isolation of the purified product.

Synthesis of stearyl palmitate, oleyl oleate, oleyl gamma-linolenate and oleyl linoleate. In like manner to the synthesis of oleyl palmitate, the synthesis of these other esters was achieved in 20% *iso*-octane/80% water (v/v) solvent system. The product was isolated after 170 min of reaction time and purified for high-resolution mass spectrometric analysis.

Mass-spectral measurements for the purified crystalline stearyl palmitate gave a molecular ion *m/z* 508, with an accurate mass of *m/z* 506.51970 (error 4.4 ppm from the calculated formula $C_{34}H_{68}O_2$, stearyl palmitate). A major fragment occurred at *m/z* 257 ($M^{+\cdot}$-251, breaking of the ester linkage). The characteristic C_nH_{2n+2} alkane fragmentation pattern was observed in the mass region below *m/z* 200 (the *m/z* 57, 71, 85 series) as expected for the stearyl and palmitate groups.

The mass spectrum of the molecular ion from the purified oleyl oleate was *m/z* 535, with an accurate mass of *m/z* 532.52021 (error 3.0 ppm from the calculated formula $C_{36}H_{68}O_2$, oleyl oleate). A major fraction occurred at *m/z* 250 ($M^{+\cdot}$-282, breaking of the ester linkage). The characteristic C_nH_{2n} alkene fragmentation pattern was observed in the mass region below *m/z* 200 (the *m/z* 55, 69, 83 series) as expected for the oleyl group.

The gamma-linolenic acid supplied by Callanish (Isle of Lewis, Scotland) as an 80% pure oil was analyzed by capillary gas chromatography on a DB225 column after conversion of the acids to their corresponding fatty acid methyl esters. The major components were *gamma*-linolenic acid (γ-C18:3 W6) 77.17% and *cis*-linoleic acid (C18:2 W6) 14.31%. The NMR spectrum of the purified product from the oleyl *gamma*-linolenate synthesis showed evidence for two ester products. The signals at 64.38 and 173.8 ppm were attributable to oleyl linoleate (see below) and those at 64.4 ppm and 170.0 ppm were attributable to oleyl *gamma*-linolenate. The signals from the two esters were not totally resolved and a separation procedure was not developed in this work. The molecular ion was *m/z* 528, with an accurate mass of 528.48872 (error 3.6 ppm from the calculated formula $C_{36}H_{64}O_2$, oleyl *gamma*-linolenate). A major fragment occurred at *m/z* 250 ($M^{+\cdot}$-278, breaking of the ester linkage). The characteristic C_nH_{2n} alkene fragmentation pattern was observed in the mass region below *m/z* 200 (the *m/z* 55, 69, 83 series) as expected for the oleyl group.

The mass spectrum of the purified oily product obtained from synthesis of oleyl linoleate gave a molecular ion *m/z* 530, with an accurate mass of *m/z* 530.50439 (error 3.6 ppm from the calculated formula $C_{36}H_{66}O_2$, oleyl linoleate). A major fragment occurred at *m/z* 248 ($M^{+\cdot}$-282, breaking of the ester linkage). The characteristic C_nH_{2n} alkene fragmentation pattern was observed in the mass region below *m/z* 200 (the *m/z* 55,69,83 series) as expected for the oleyl group.

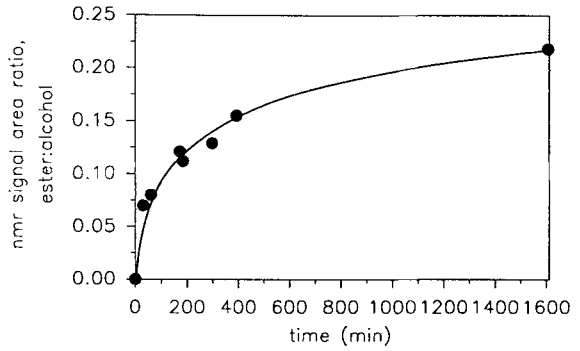

FIG. 6. Oleyl *gamma*-linolenate synthesis in 20% *iso*-octane/80% water (v/v) solvent system at 37°C; ^{13}C NMR determination. (Initial ratio of acid:alcohol = 1:5.)

Enzyme deactivation test. Twenty μL of the aqueous phase separated from the *C. cylindracea* lipase-catalyzed esterification of oleic acid with oleyl alcohol in 20% *iso*-octane/80% water (v/v) solvent system after 20 hr' reaction time at 37°C was used to catalyze the hydrolysis of 4-nitrophenyl acetate in tris buffer (pH 7.5) at 37°C. This activity was compared with that of 20 μL of the aqueous enzyme phase removed from the esterification mixture at zero time. Activity of the enzyme after the 20 hr' reaction was 1.5 times the initial activity.

Substrate preference. C. cylindracea lipase-catalyzed esterifications were achieved with both saturated and unsaturated fatty acids and alcohols. Oleyl oleate synthesis proceeded more rapidly than did oleyl palmitate synthesis (Fig. 5). Osterberg *et al.* (11) have previously noted a preference of lipases from *Rhizopus* strains for specificity in double bond positions rather than to the degree of unsaturation. The double bond was thought to restrict rotation, thereby altering the conformation of the substrate and hence the binding strength for the enzyme-substrate complex. The oleyl *gamma*-linolenate synthesis (Fig. 6) proceeded more slowly than either of the syntheses shown in Figure 5, but because the reactant was a mixture of at least two fatty acids and the resolution between the ^{13}C signals was incomplete, full interpretation of these results is not possible.

Stearyl palmitate synthesis was achieved, in rapid and near quantitative yield, by the *C. cylindracea* lipase action on stearyl alcohol and palmitic acid. This result illustrates the efficiency of the enzyme for the production of saturated esters. With this method, stearyl esters could be produced industrially on a continuous cycle that included crystallization to remove the product.

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