1-Monoglyceride Production from Lipase-Catalyzed Esterification of Glycerol and Fatty Acid in Reverse Micelles

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Glycerol-fatty acid esterification has been conducted with lipase from *R. delemar* in water/AOT/isooctane reverse micellar media, with the major product being 1-monoglyceride, a useful food-emulsifier. 1,3-Diglyceride was also synthesized, but to a much lesser extent. For a given set of initial conditions, the reaction productivity, measured in terms of the initial product formation rate, V_0, and the final or equilibrium concentration of product, is optimal for a particular concentration of each surfactant, fatty acid, glycerol, and water. Many of these optimal values correlate well with a "critical" region on the phase diagram. Also, results indicate lipase-catalyzed esterification stops due to the achievement of kinetic equilibrium except for a few cases where enzyme deactivation is severe. Dynamic light scattering was employed to examine the influence of water, glycerol, and fatty acid on micellar and interfacial structure. Results from this technique indicate enzyme kinetics are linked to interfacial phenomena and the presence of substrates at the interfacial region.

Key words: lipases • reverse micelles • dynamic light scattering • glycerol-fatty acid esterification

INTRODUCTION

The enzyme lipase (EC 3.1.1.3) has proven to be useful in a myriad of applications in industry. Moreover, the utility of enzymes such as lipase have been found in their ability to catalyze useful reactions in mild conditions at high conversions and selectivities due, in part, to their regio- and stereospecificities. Of the numerous enzymes in existence, lipases are particularly desirable biocatalysts to work with because of their relative inexpensiveness and commercial availability as well as their ability to operate in a broad pH range without the need of cofactors. A comprehensive review of various industrial applications of lipases has recently been written.[22] Of particular interest is the biocatalysis of lipase in nearly anhydrous organic media, as pioneered, for example, by A. M. Zlibanov's research group.[44,45] This technology allows lipase to act upon lipophilic substrates that cannot solubilize in aqueous media and to catalyze esterification of acids and alcohols since water, a product of this reaction, is present only in small quantities in order to solvate the charged groups of the enzyme. Most research for lipase biocatalysis in organic media has been performed with it immobilized (adsorbed) on celite or other suspension material or entrapped in gels.[22] Applications of this technology include resolution of menthol stereoisomers through fatty acid esterification and triglyceride transesterification,[25] production of geranic acid esters[26] which are important components of perfume, synthesis of useful food emulsifiers such as fatty acid-sugar esters,[40,41] and alteration of cooking oils through interesterification with fatty acids[10,37,39,43] and transesterification with other triglycerides.[31] Our research effort has focussed upon esterification between fatty acids and alcohols, particularly glycerol in this report. These reactions can produce useful materials such as flavor enhancers (e.g., benzyl and cinnamic alcohol esters),[1] food emulsifiers (e.g., 1-monoglycerides),[24] and texturizing agents in cosmetic products (e.g., ethylene glycol-stearic acid mono- and diesters).[18] Also by employing 1,3-positional specific and nonspecific lipases at different steps of glyceride synthesis, triglycerides of unique structure such as POP and POS can be tailor-made.[31]

In particular, a problem with employing polyols such as glycerol in esterifications is their poor solubility in apolar organic solvents.[37] Ergan et al.[6] resolved this problem by solubilizing glycerol in the liquid substrate oleic acid; however, most fatty acids exist as solids in the operational temperature range of lipases, thus limiting the method's utility. Also, Hoq et al.[17] have achieved glyceride synthesis using a bioreactor which has lipase adsorbed onto a membrane between a fatty acid-laden organic phase and a glycerol/aqueous phase. Although conversions were reasonably high and enzyme stability retained, a major problem existed with enzyme desorption from the membrane. Another solution to the problem is the encapsulation of large quantities of glycerol into reverse micelles, which are nanometer-sized aqueous or polar dispersions in apolar media of which the interface is controlled by surfactants/cosurfactants. The properties of reverse micellar media and their employment in enzymology have been thoroughly reviewed.[5,28,38] Compared to the immobilization techniques mentioned above, use of reverse micelles have many other advantages, including easier media and enzyme preparation, less mass-transfer limitations (due in part to its large degree of interfacial area and rapid rate of micellar breakage/coalescence), and simpler control

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and monitoring of the water content. However, disadvantages exist, particularly for process development. Moreover, a reactor design for operating the reaction in semibatch or continuous mode and allowing for the separation of substrates, products, and enzyme has not been successfully developed. Lipase catalysis in reverse micelles has been shown feasible for these reactions: hydrolysis,[11,15] fatty acid-alcohol esterification,[12,36] trans- and interesterification of triglycerides,[2,16] and synthesis of glycerides.[7,12,32] In this report, we have thoroughly investigated glycerol–fatty-acid esterification by lipase from *R. delemar* (a 1,3 positional specific lipase) in water/AOT/isooctane reverse micellar media for the purpose of producing 1-monoglycerides and have determined the effects of interfacial phenomena on this reaction.

MATERIALS AND METHODS

Materials

AOT (ca. 99% pure) was purchased from Aldrich (Milwaukee, WI) and used without further purification. Our results using UV-vis spectrophotometry, conducted on a Hewlett-Packard (Palo Alto, CA) model 8451A spectrophotometer, as a method of comparison show that Aldrich's product is similar in purity to the purified AOT used in our previous work.[12] Two lipase types were employed for catalyzing reactions and used without further purification: the first, from *R. delemar,* was purchased from Fluka (Ronkokoma, NY); the second, from *C. cylindracea,* was purchased from Sigma (St. Louis, MO). All other reagents used were of high purity (>99%) and not purified further. Deionized water was used throughout.

Methods

Glycerol–fatty-acid esterification was carried out as follows. First, glycerol-containing reverse micellar media was formed by mixing glycerol and an isooctane solution containing AOT and fatty acid at typical concentrations of 200 and 100 m*M*, respectively, with the assistance of agitation/sonification. Then an aliquot of a lipase solution in 50 m*M* phosphate buffer, pH of 6.89 ±0.05, was injected into the media to initiate the reaction (The addition of lipase from *R. delemar* to aqueous media for concentrations employed here slightly increased bulk pH—by less than 0.1 units—while the addition of lipase from *C. cylindracea* had no such effect on bulk pH.). Gentle agitation for 1–2 min in most cases clarified the solution, indicating the incorporation of water and enzyme into reverse micellar media. (At conditions near phase boundaries, solutions when occasionally agitated clarified after a few minutes to an hour.) At selected times, aliquots of media were withdrawn for kinetic analysis. Analysis of substrates and products was achieved by employing capillary gas chromatography with trimethylsilane (TMS) ether derivitization and an internal standard, similarly to that described in our previous report.[12] Besides providing for separation and quantification of various types of fatty acids, mono-, di-, (and tri-) glycerides, the methodology permitted resolution of positional isomers, such as 2- and 1-monoglycerides. Enzyme quantities are reported here in terms of "Units" of activity, which are defined as lipase's ability to esterify 1 m*M* lauric acid with *n*-butanol per hour in reverse micellar media at these initial conditions: w_0 = [H_2O]/[AOT] = 9.23 (mol/mol), [lauric acid] = [AOT] = 100 m*M*, [*n*-butanol] = 0.4 m*M*, temperature = 24°C, and aqueous pH 6.88. Kinetic trends and results reported in this article have been shown to be repeatable, with errors in substrate/product concentration being within five percent of the values indicated.

The properties of water (50 m*M* phosphate buffer)/AOT isooctane reverse micellar media containing glycerol and lauric acid were examined through composition of phase diagrams and dynamic light scattering (DLS) measurements. Phase boundaries were estimated from titrations using phosphate buffer, glycerol, and a stock solution of AOT and lauric acid in isooctane; the titration endpoints were determined through visualization of the cloudiness/clarity transition between two-phase and reverse micellar monophasic media. The equipment used for DLS was described in our previous article.[12] DLS sample preparation included filtration (0.2 μm) followed by centrifugation; samples contained no enzymes. With DLS, the average size or hydrodynamic radius (R_h's) were determined from the Stokes–Einstein equation:

$$R_h = kT/6\pi\mu D_0$$

where k is the Boltzman constant; T the absolute temperature; μ the solution viscosity; and D_0 the average translational diffusion coefficient at an infinitely dilute concentration of dispersions. Equation (1) holds true for spherically shaped aggregates only, of which classification reverse micelles are members.[5] Parameter D_0 was determined from measurements of the apparent diffusion coefficient, D_{app}, at various concentrations of micellar aggregates dispersed in the organic continuum. Experimentally, a stock solution of reverse micellar media was successively diluted with isooctane, with D_{app} being measured at a scattering angle, θ, of 90° for each diluted sample using the method of cumulants[23] similarly to that described in our previous work.[12] A plot of D_{app} against the concentration of dispersions—or equivalently ϕ, the volume fraction of dispersed phase (here, being approximated by the volume fraction of phosphate buffer plus glycerol)—is linear near the ordinate and can be described by the following relationship[4]:

$$D_{app} = D_0(1 + \alpha\phi) \quad (2)$$

where α is an important parameter quantifying interaction between dispersions. Indeed, our data obeyed the

straight-line relationship of Eq. (2) with correlation coefficients in most cases being greater than 0.99. Many of the DLS results shown have been verified with duplicate trials, with differences between duplicate measurements being small (within 5%). Error bars, determined primarily from the precision of the fit of Eq. (2) to data, are provided in the figures.

RESULTS

Glyceride Synthesis in Reverse Micelles

Figure 1 shows the phase boundaries for monophasic water/AOT/isooctane reverse micellar media at fixed temperatures containing the substrates glycerol and lauric acid. Gibb's phase rule indicates at the boundary between two phases at constant temperature, the number of degrees of freedom, ν, is equal to the number of components, n, which here is five. To decrease ν to 3, the ratio lauric acid/AOT/isooctane was fixed at each of various ratios. Using this value of ν, a triangular phase diagram with [H_2O], [glycerol], and [AOT] (or [lauric acid]) at the vertices could have been formed. However, the media used in our kinetic experiments contained AOT at low concentrations (50–300 mM). In this low concentration range, we found the phase boundary did not change significantly when [AOT] was varied and the molar ratio of dispersed phase-to-AOT held constant. In addition, this ratio is of importance here since it dictates micellar size. These reasons convinced us to transform our triangular phase diagram to one in terms

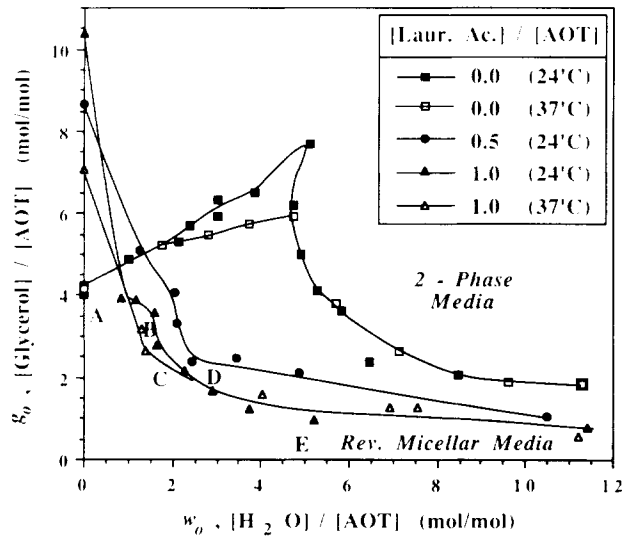


Figure 1. Phase boundaries for reverse micellar phase of media water/glycerol/AOT/lauric acid/isooctane at various combinations of temperature and lauric acid/AOT molar ratio. Area below the boundary indicates region for existence of one-phase reverse micellar media; above it the corresponding media is two-phase. AOT concentrations (with respect to isooctane) used in the titrations were: 100 mM for lauric acid–AOT ratios of 0 and 1 and 200 mM for a ratio of 0.5; see text for more detail. Points A, B, and C are explained in the text.

of two variables: the water–AOT molar ratio, w_0, and the glycerol–AOT molar ratio, g_0, since water and glycerol are the two components of the dispersed phase. Figure 1 depicts phase boundaries for various combinations of temperature and lauric acid/AOT molar ratio. The area below a phase boundary represents the region where monophasic reverse micellar media exists; above it, two-phase and nonmicellar medias are produced.

Figure 1 shows that the shape of the phase boundaries in the presence of fatty acids is generally hyperbolic; moreover, when a large quantity of water is encapsulated in reverse micelles, the media can incorporate less glycerol and vice versa, which suggests that water and glycerol compete for solubilization in the inner core of reverse micelles. Indeed, light scattering results support this observation (see ref. 31 and the results below). Also of interest is the phase boundary for the case where no lauric acid is present, which illustrates the solubilization of glycerol actually increases with water content for $w_0 < 5$, a result that contradicts the trend just discussed. In addition, deviations from the hyperbolic shape of the boundaries of lauric acid-containing media are present (especially at 24°C) at glycerol/water ratios near one (e.g., near point B for the [lauric acid]/[AOT] = 1.0 case). This region of the phase diagram, to be referred to as the "critical region" in this report, will have importance in kinetic results, as will be demonstrated. Figure 1 also suggests in this region the effect of temperature is quite significant, with the micellar phase region area being decreased at the higher temperature.

Titrations have also been performed to determine the maximum solubility of lauric acid in water/glycerol/AOT/isooctane reverse micellar media at 24°C; these results indicate in general fatty acid solubility is higher when the media contains more water and less glycerol. For example, at the conditions of point A of Figure 1, where only glycerol is present, the maximum allowable lauric acid/surfactant molar ratio for micellar media formation is 1.9, whereas the conditions specified by point E allow a ratio of >5.0. The lowest solubilization of fatty acid (points B–D: at lauric acid/AOT maximum ratios between 0.6 and 1.7) occurs for media composition in the above-mentioned "critical region." Possible explanations for these observations as well as their significance will be discussed below.

The region under the phase boundaries of Figure 1 represents the possible media compositions that can be used in conducting glyceride synthesis in reverse micelles. Figure 2 illustrates the results of glycerol–lauric acid esterification employing one such set of conditions (i.e., temperature, surfactant concentration, and initial concentrations of lauric acid, glycerol, i.e., g_0, and water, i.e., w_0) and the biocatalyst lipase from *R. delemar.* As can be seen in Figure 2, lauric acid, the limiting substrate, decreased in concentration until it reached a final constant value. Thus, the reaction did not go to completion, but stopped at ca. 60% conversion. The causes of this stoppage or "equilibrium" will be ex-

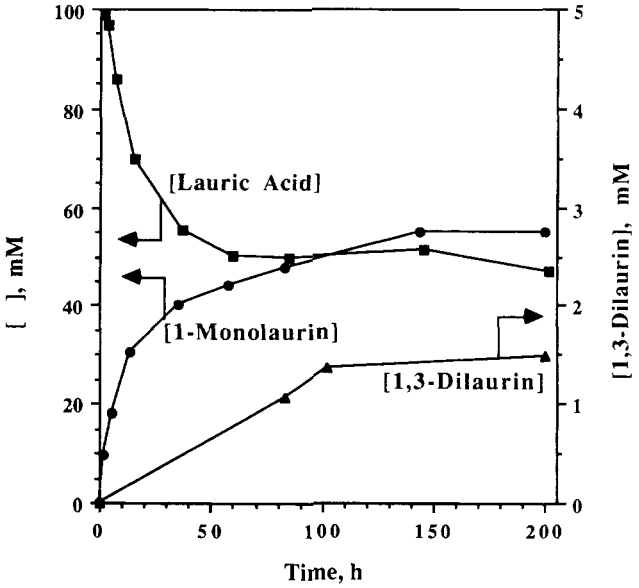


Figure 2. Concentration profiles of substrate and products for esterification of lauric acid and glycerol by lipase from *R. delemar* in AOT reverse micelles. Initial conditions are temperature of 24°C; [AOT] = 200 m*M*; $w_0 = 1.5$; $g_0 = 2.5$.

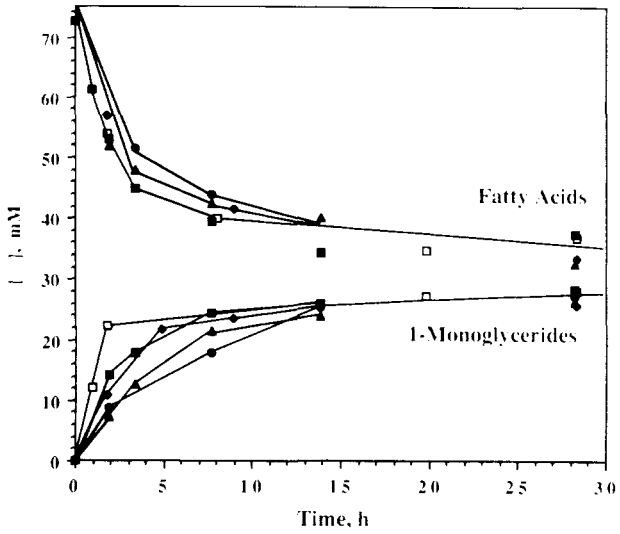


Figure 3. Effect of fatty acid type on glyceride synthesis. Each set of fatty acid/1-monoglyceride profiles corresponds to a separate experiment, each of which was performed at the following initial conditions: temperature of 24°C; [AOT] = 200 m*M*; and $w_0 = g_0 = 2.0$. The symbols show the following fatty acid/1-monoglyceride types: (—■—) caprylic acid/1-monocaprylin, (—◆—) lauric acid/1-monolaurin, (—▲—) myristic acid/1-monomyristin, (—●—) palmitic acid/1-monopalmitin, and (—□—) oleic acid/1-monoolein. The units of enzyme employed were the same for each experiment shown.

plored in detail below. The major product formed was the monoester, 1-monolaurin; only a small quantity of the diester (1,3-dilaurin) was produced. The conformation of these two products reflects the 1,3-positional specificity of lipase from *R. delemar;* the corresponding esters 2-monolaurin and 1,2-dilaurin were detected only at trace concentrations after a hundred hours. As noted above, the major product, 1-monoglyceride, is an important food emulsifier.

The use of other fatty acid types has been explored with glyceride synthesis in reverse micelles. Figure 3 displays results from a set of five experiments conducted in identical fashion at identical conditions, but with each having a different fatty acid type ranging in size from 8 carbons (caprylic acid) to 18 carbons (oleic acid). From Figure 3, it can be seen that approximately an equal portion of each fatty acid was consumed and the amount of product formed at "equilibrium" for each experiment was the same, even though initial rates differed. Two corollary experiments were performed with lipase-catalyzed glyceride synthesis in reverse micelles that yielded equivalent results. First, a reaction was conducted with media initially containing six different fatty acids at equimolar concentrations. The amount of each fatty acid consumed as well as the final concentrations of 1-monoglycerides were nearly identical (within 5% of each other). Second, a reaction media containing three fatty acids present at a molar ratio of A:B:C produced corresponding final 1-monoglyceride concentrations at the same ratio. Both of these experiments further indicate the extent of lipase-catalyzed glycerol esterification is not related to fatty acid type, but to substrate concentration and media composition. The difference in initial rates in Figure 3 mirrors the enzyme's fatty acid substrate specificity, as was the case for fatty acid-alcohol esterification in reverse micelles.[12] More specifically, the order of fatty acids in terms of reactivity, as measured by V_0, the initial rate, is:

oleic acid > caprylic acid > myristic acid =
lauric acid > stearic acid = palmitic acid

which nearly matches the substrate specificity reported in the literature[19,42] with the exception of caprylic acid although differences in relative rates here are less than those reported.

Figure 4 shows the effect of initial fatty acid concentration, $[FA]_0$, on production of 1-monolaurin at "equilibrium" for various initial concentrations of water and glycerol, as expressed through the values of w_0 and g_0 indicated in the legend. (The temperature and surfactant concentration were identical for all cases.) For each experiment shown, an optimal fatty acid concentration, $[FA]_{opt}$, existed. From Figure 4, certain trends concerning $[FA]_{opt}$ can be observed. For reverse micellar media high in glycerol concentration ($g_0 > w_0$), the value of $[FA]_{opt}$ was relatively low; 1-monolaurin production decreased to a large extent when higher fatty acid concentrations were employed (e.g., for $w_0 = 2/g_0 = 3$). Moreover, when $w_0 > g_0$, not only is $[FA]_{opt}$ larger, but in general the production of 1-monoglyceride is higher. The initial rate of product formation, V_0, however, was found to be inversely related to $[FA]_0$ for each w_0/g_0 case investigated. Note also that in two cases: $w_0 = 2/g_0 = 3$ and $w_0 = 3/g_0 = 2$, data is reported for reactions taking place in biphasic media and trends occurring in reverse micellar media (i.e., to the left of the perpendicular

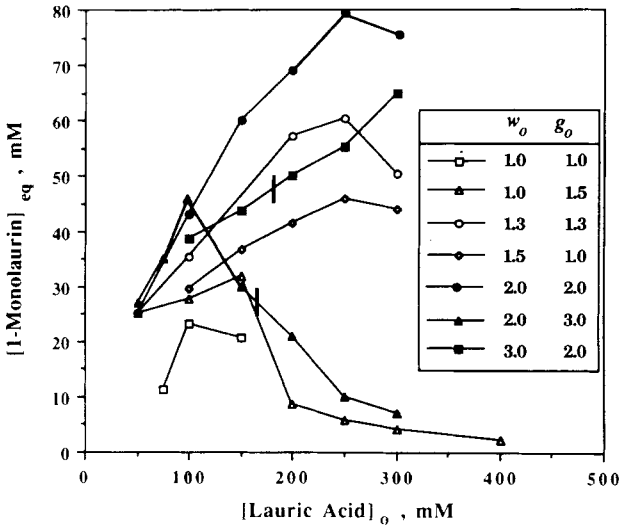


Figure 4. Effect of initial fatty acid concentration, $[FA]_0$, on lauric acid-glycerol esterification at 24°C. Plotted is the final or "equilibrium" amount of product, 1-monolaurin, synthesized per given initial concentrations, indicated by the abcissa value of initial lauric acid concentration and the corresponding values of w_0 and g_0 listed in the legend, with [AOT] = 200 m*M*. Lines drawn perpendicular to $w_0 = 3/g_0 = 2$ and $w_0 = 2/g_0 = 3$ curves represent phase boundaries; moreover, reverse micellar media is produced only for $[FA]_0$ values less than the one indicated by the line.

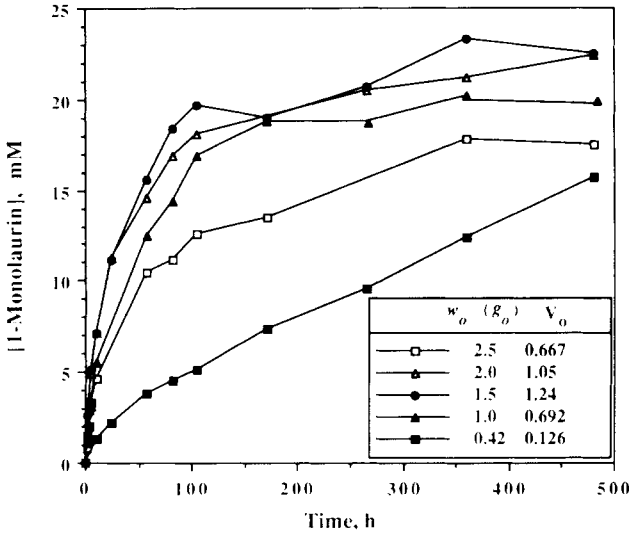


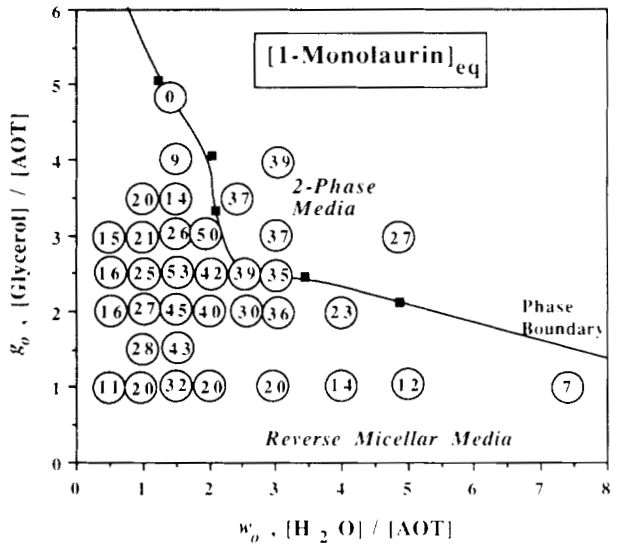
Figure 5. The effect of AOT concentration on lauric acid-glycerol esterification at 24°C. Initia conditions are [lauric acid]$_0$ = 100 m*M*; [glycerol]$_0$ = [H_2O]$_0$ = 125 m*M*. [AOT] is indicated by values of $w_0(g_0)$ listed in legend. Also, the legend contains values of V_0, the initial rate of product (1-monolaurin) formation in mM/h for each trial. The units of enzyme employed were the same for each trial.

lines) continue in the multiphase region. The results of Figure 4 will be discussed below.

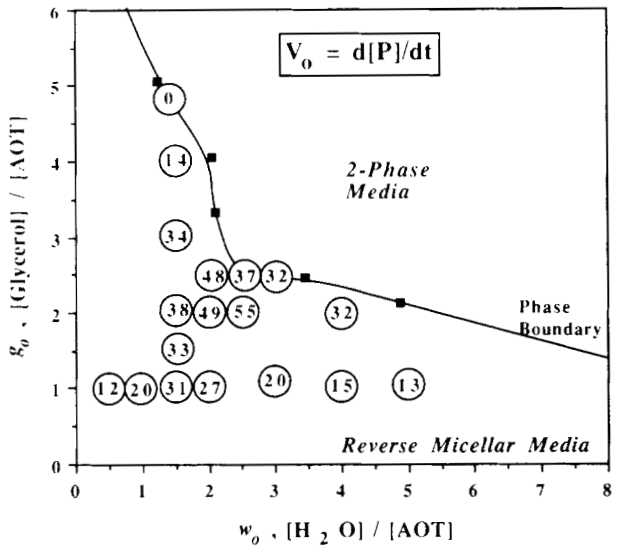
In our previous article,[12] it was illustrated that an optimal overall water-AOT mole ratio, obtained by varying the surfactant concentration, existed for maximizing V_0 for lipase-catalyzed fatty acid-alcohol esterification in reverse micelles, while the final "equilibrium" concentrations of substrates and product were not significantly affected. Figure 5 depicts a similar trend for glycerol-lauric acid esterification. In the five experiments shown here, all initial conditions and concentrations were identical, except for the surfactant concentration, which altered the value of initial w_0 (and g_0) as demonstrated in the legend (values of w_0/g_0 for "filled" and "unfilled" reverse micelles, referring to protein-containing and protein-absent dispersions, respectively, may differ). The initial rate values, also listed in the legend, suggest an optimal value of surfactant concentration (producing w_0/g_0 values of 1.5) for the initial conditions employed. However, unlike fatty acid-alcohol esterification, the "equilibrium" concentrations of substrates and products in these cases was not the same, but mirrored the trend in V_0 values. The difference, though significant, is not large, especially when compared to the difference between V_0's, as the final concentration of 1-monolaurin varied only between 16 and 23 m*M*. Note for the case $w_0(g_0) = 0.42$, conversion still occurred after 500 h, indicating long-term enzyme activity retention in reverse micelles for this set of conditions. Reasons for the occurrence of the optimal [AOT] are yet unknown.

Many experiments were performed at different water and glycerol concentrations employing fixed values of temperature (24°C), [AOT] (200 m*M*), and [lauric acid]$_0$ (100 m*M*). The results are summarized in Figure 6. Figure 6(A) lists the amount of monoglyceride produced at "equilibrium" for the initial values of w_0 and g_0 corresponding to the center of each circle (e.g., 14 m*M* of 1-monolaurin are produced when $w_0 = 4$ and $g_0 = 1$ initially), while Figure 6(B) depicts initial product formation rate values. Note that operation in one-phase reverse micellar media is restricted to combinations of initial w_0 and g_0 values existing below the phase boundary. Figure 6 indicates the productivity of glyceride synthesis, as measured by 1-monoglyceride (as well as 1,3-diglyceride) production and the initial rate, is greatest near the "critical region" of the phase diagram; for example, at $w_0 = 1.5$ and $g_0 = 2.5$, ca. 60% of fatty acid is converted into 55 m*M* 1-monolaurin and 2 m*M* 1,3-dilaurin, which is about the highest conversion achieved for this combination of surfactant and initial fatty acid concentrations and temperature (see Fig. 2). These results correlate high production for this reaction directly to the composition and structure of the reverse micellar media. Notice from Figure 6(A) that conversions do not improve significantly upon operation beyond the micellar phase boundary.

In our previous work,[12] we reported glycerol-fatty acid esterification was quite sensitive to temperature, where production of 1-monoglyceride was highest at 24°C, decreased eight-fold at a slightly higher temperature (28°C), and was very small at 37°C, a near-optimal temperature for activity of lipase from *R. delemar*.[20] To help determine whether enzyme deactivation was causing reaction stoppage at this high temperature (as well as at 24°C), an experiment was conducted using glycerol–lauric-acid synthesis at fixed initial conditions. For both temperatures, 38 and 24°C, three trials were run, with



(A)



(B)

Figure 6. A summary of lauric-acid–glycerol esterification in reverse micelles superimposed on the phase diagram for the following conditions: temperature of 24°C; [AOT] = 200 m*M*; [lauric acid]$_0$ = 100 m*M*; w_0, g_0 values indicated by corresponding abcissa and ordinate values of the center of circles. Numbers shown in circles are (A) values of [1-monolaurin]$_{eq}$ produced in m*M* units; (B) initial rates of product (1-monolaurin) formation, V_0's, in units of mM/h/(Units lipase/mL media) × 10, where enzyme Units are defined in the text.

each employing a different enzyme concentration. Figure 7 illustrates the results. Figure 7 depicts at 38°C the production of 1-monolaurin stops after only ca. 2 h for all three cases and that final production was higher for the larger initial enzyme concentration. These results indicate lipase deactivates rapidly at this temperature in water/glycerol/AOT/lauric acid/isooctane reverse micellar media for this set of conditions. However, the trials at 24°C approach nearly the same endpoint in terms of the concentrations of fatty acid, 1-monolaurin (as shown in Fig. 7), and 1,3-dilaurin, and initial rates were directly proportional to the enzyme concentration. These encouraging results suggest enzyme activity at room temperature (at least for this set of conditions) is strongly retained and indicate reaction termination is due to the onset of "equilibrium." Figure 7 also illustrates the effect of rapid agitation on the reaction. The results demonstrate agitation did not alter the final "equilibrium" endpoint of the reaction and only slightly accelerated the rate. In addition, Han and Rhee[11] have demonstrated agitation causes long-term lipase deactivation in reverse micelles. Both of these results indicate agitation is not necessary or desirable.

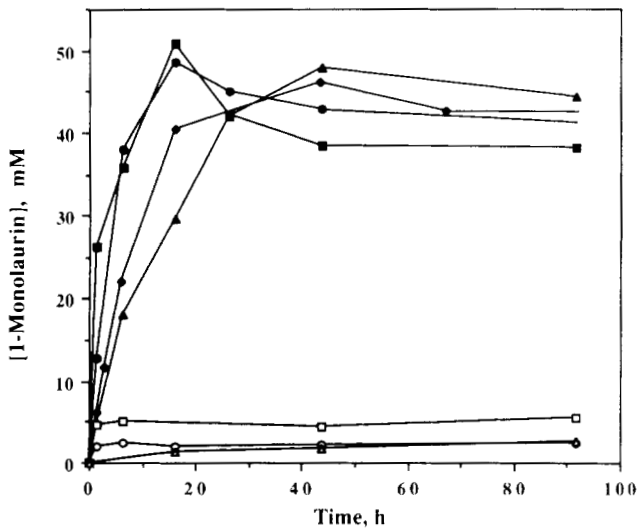


Figure 7. The effect of enzyme concentration and agitation on lauric acid-glycerol esterification at 24°C (darkened symbols) and 38°C (outlined symbols). Initial concentrations are [AOT] = 200 m*M*; [lauric acid]$_0$ = 100 m*M*; w_0 = 2.0, g_0 = 2.5. The symbols show (in Units/mL): (—■—, —□—) 7.07, (—●—, —○—) 1.42, (—▲—, —△—) 0.71, and (—◆—) 0.71 with agitation.

Dynamic Light Scattering

The results of Figures 4–6 have provided the motivation to explore how glycerol, fatty acid, and water interact in reverse micellar media. The tool chosen to obtain this information was dynamic light scattering (DLS). From DLS, we were able to obtain three important measurements of the media behavior: the average size or hydrodynamic radius (R_h) of the micellar dispersions, an index of polydispersity for micellar size, and the magnitude of interactions between reverse micellar aggregates (the parameter α). The indices of polydispersity in most cases were low (less than 0.15), indicating that the populations of reverse micelles formed here are quite monodisperse.

Figure 8 displays measurements of the two parameters, R_h and α, for reverse micelles as a function of g_0 at w_0 = 1.5 for media in the presence/absence of fatty acid and at both 24 and 38°C. Figure 8(A) illustrates for all cases the micellar radii increased with g_0, which supports the claim that glycerol primarily solubilizes with

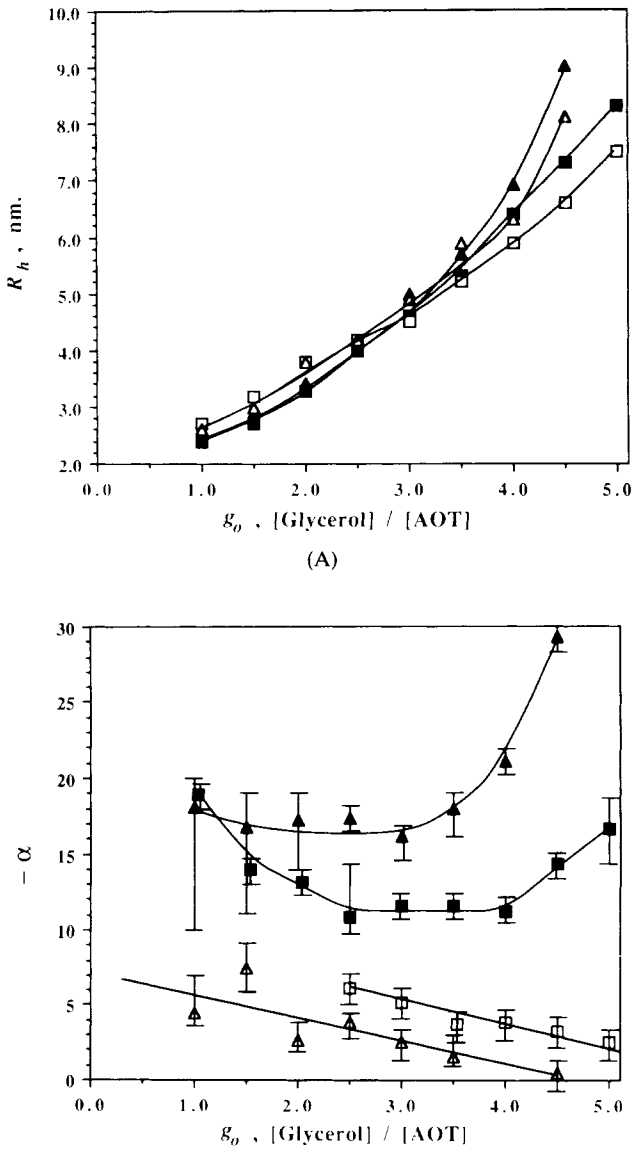


Figure 8. Effect of glycerol content, g_0, on: (A) Hydrodynamic radii, R_h's, and (B) micellar interaction parameters, α's, for water/glycerol/AOT/lauric acid/isooctane reverse micelles at (-■-, -□-) 25°C and (-▲-, -△-) 38°C for w_0 = 1.5. Darkened symbols show no lauric acid present; outlined symbols show [lauric acid]/[AOT] = 0.5 (mol/mol). Errors for A are indicated by the size of the symbols.

water in the inner core of reverse micelles. Figure 8 also depicts an interesting relationship between fatty acid presence and micellar size. For $g_0 > 2.5$, the addition of fatty acid decreased micellar size; however, for lower g_0 values, the presence of fatty acid caused a slight increase in R_h. In terms of micellar size, an increase in temperature had no significant effect except at high glycerol-surfactant ratios ($g_0 > 3.0$). Figure 8(B) indicates in all cases the value of α, the interaction parameter, was negative, suggesting the nature of the interactions was attractive (perhaps due to van der Waals forces). The presence of glycerol increased the magnitude of interactions significantly (the value of α for water/AOT/heptane reverse micellar media is approximately -2[35]), but when fatty acid was added, $|\alpha|$ was reduced dramatically. The relationships between $|\alpha|$, temperature and g_0 in the absence of fatty acid strongly agree with the work of Fletcher et al.[8] who investigated glycerol/AOT/heptane micellar media. Thus, the presence of glycerol and/or fatty acid has a strong effect on the behavior of the micellar media, as will be discussed below. The decrease in $|\alpha|$ with increasing g_0 for fatty acid-containing media shown in Figure 8(B) is an interesting result which cannot yet be explained.

The effect of w_0 on R_h and α for two fixed values of g_0 at 24°C are presented in Figure 9. Figure 9(A) illustrates at both g_0 values the micellar size changed very little

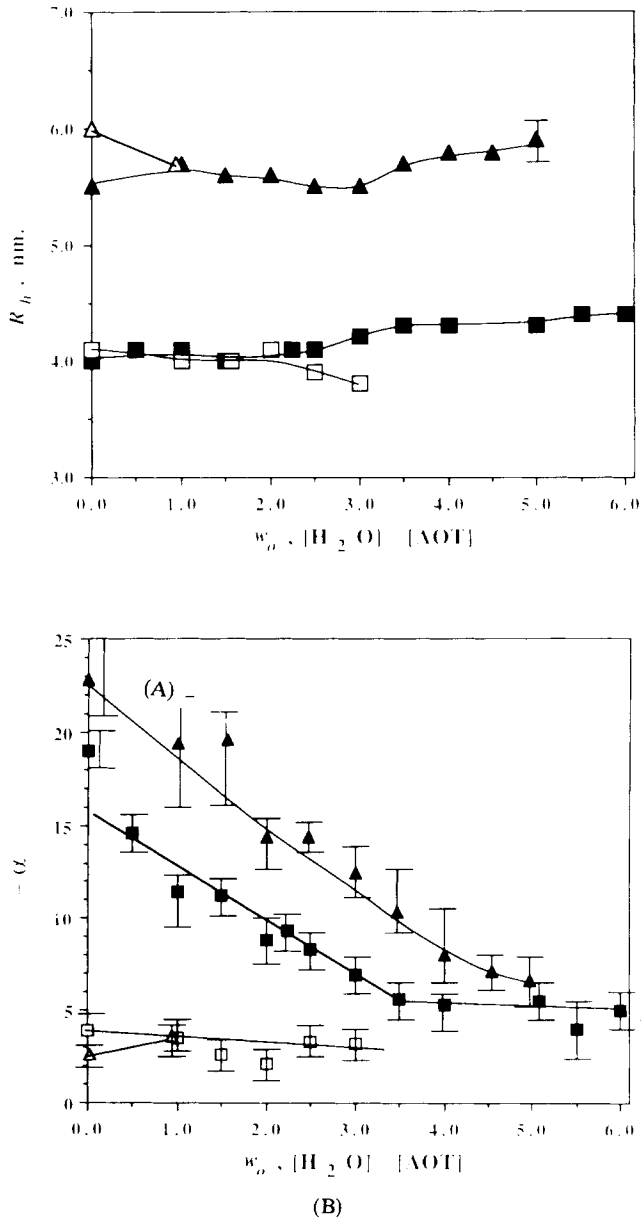


Figure 9. Effect of water content, w_0, on: (A) hydrodynamic radii, R_h's, and (B) micellar interaction parameters, α's, for water/glycerol/AOT/lauric acid/isooctane reverse micelles at 25°C with g_0 values: (-■-, -□-) 2.5, (-▲-, -△-) 4.0. Darkened symbols show no lauric acid present; while outlined symbols show [lauric acid]/[AOT] = 0.5 (mol/mol).

with w_0; larger differences were expected in light of micellar size data obtained for water/AOT/isooctane micellar media.[12] The addition of fatty acid did not alter R_h significantly either. Figure 9(B) illustrates the interesting trend that the addition of water strongly decreased interactions between micelles. Note also that the interactions at the higher g_0 value were larger, which matches the data from Figure 8(B) for $g_0 > 2$. As before, the addition of fatty acid decreased the magnitude of micellar interactions, but for fatty acid-containing micellar media $|\alpha|$ did not change significantly with w_0.

The influence of fatty acid concentration on micellar properties is shown explicitly in Figure 10 for two fixed combinations of water and glycerol content: $w_0 = 3/g_0 = 2$ and $w_0 = 2/g_0 = 3$. For both cases, Figure 10 demonstrates only a small amount of fatty acid can influence the media significantly, as $|\alpha|$ was decreased by a factor of 2 for a fatty-acid–AOT molar ratio of only 0.125. It can be seen for both cases that R_h is insensitive to the fatty acid content. These results will also be discussed below.

DISCUSSION

Glycerol-fatty acid esterification occurs readily in reverse micelles. With lipase from *R. delemar,* conversions reached as high as 50–60%, with 1-monoglycerides being the major product. 1,3-Diglycerides were also produced, but at relatively low amounts; only trace amounts of 2-monoglycerides and 1,2-diglycerides were detected after long time periods. Fletcher et al.[7] performed the same reaction as us, but employed lipase from *C. viscosum.* The reported conversions were as high as 80–90%,

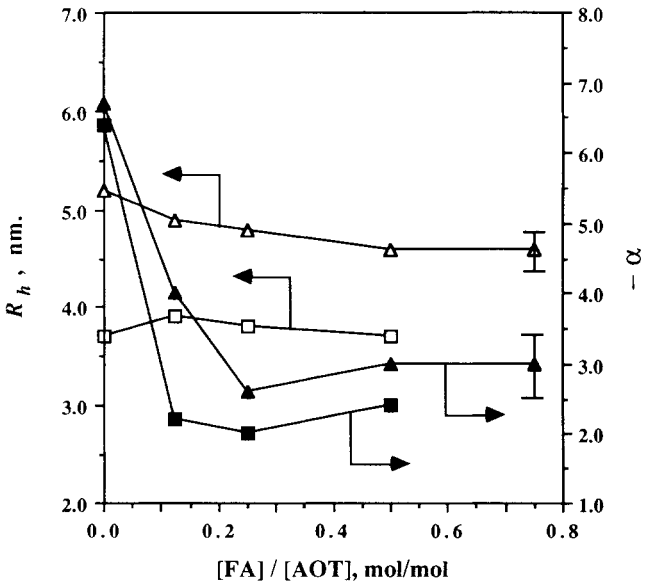


Figure 10. Effect of lauric acid/AOT molar ratio on the hydrodynamic radius, R_h (outlined symbols), and the interaction parameter, α(darkened symbols), for water/glycerol/AOT/lauric acid isooctane reverse micelles at 24°C: (—□—, —■—) $w_0 = 3.0$, $g_0 = 2.0$; (—△—, —▲—): $w_0 = 2.0$, $g_0 = 3.0$.

but the lipase employed was not 1,3-positional-specific and thus was able to utilize 1.5 times as many free glycerol hydroxyls. As was the case for us, their major product was monoglyceride, but their product mixture probably contained both 1- and 2-monoglycerides. (We have performed the same reaction with lipase from *C. cylindracea,* which also has no positional specificity. The production, which was quite low due to enzyme deactivation, included both 1- and 2-monoglycerides at a ratio slightly greater than 2:1.) It is the former positional monoglyceride monomer which is the more valuable product as a food emulsifier.

Thus, the feasibility of glyceride synthesis in reverse micelles has been shown, but many issues need to be resolved before this technology can be applied to pilot plant and large-scale production. One such issue is the stability of lipase in this media. Immobilized lipase has shown strong activity retention in organic media.[44] Similarly, Fletcher et al.[7] reported the activity of lipase from *C. viscosum* in AOT reverse micelles remained high for several days. Indeed, the data shown in Figure 5 for the $w_0(g_0) = 0.42$ case concurs. But in contrast, we found the stability of lipases from both *C. cylindracea* and *R. delemar* to be poor in reverse micellar media,[12] especially for certain conditions (as will be discussed in more detail below). This issue has not yet been fully resolved. Another problem is surfactant type. To explain, since AOT is unsuitable for many food-related applications and its removal from the media can be difficult,[34] other micellar media containing "edible" surfactants have been investigated. Results demonstrated these media to be inferior to AOT-based media in terms of production,[32] and in some cases the surfactant underwent side reactions.[16] A third problem is the scale-up of reverse micellar media. Attempts have been made to develop semibatch and continuous reactor design for reverse micellar media,[29] but no breakthroughs have occurred. Also, in scaling batch size, the issue of operating with reverse micelles at high enzyme concentration has not been investigated thoroughly. However, recovery of enzyme activity has been shown to be feasible through extraction steps.[35] We are currently investigating the recovery of substrates and products from the final reaction media employing an extraction technique for the removal of unreacted glycerol and AOT and adsorption liquid chromatography for the separation of the remaining fatty acid substrate and products.

With fatty-acid–alcohol esterification, we have demonstrated the importance of having substrates at the interface, the apparent site of reaction for lipase, to maximize the rate of production.[12] Our results with glyceride synthesis also illustrate the significance of interfacial phenomena on productivity. Figure 6 strongly correlates productivity with the above-mentioned "critical" region of the phase diagram. Dynamic light scattering (DLS) was employed as a tool for exploring the effects of glycerol, water, and fatty acid on the behavior of reverse micelles particularly for this region. (Note

the influence of lipase on micellar media could not be determined by DLS here due to its low overall concentration.) An investigation of the effect of products on micellar behavior has yet to be completed. Figure 8 illustrates the effect of glycerol and fatty acid on micellar radii and the magnitude of micellar interactions. Figure 8(B) indicates glycerol's presence strongly increases the degree of micellar interactions and hence the "fluidity" of the interface[9,27] (meaning the interface is less structured, its curvature lessened and surfactant translational diffusion reduced). We believe this effect, which is similar to the one induced by alcohols/cosurfactants with small hydrocarbon tails such as butanol,[27] indicates the presence of glycerol at the interfacial region. This is consistent with recent FTIR spectroscopy results[13] which suggest glycerol's presence near the surfactant head groups, as noted by the latter's ability to disrupt the hydrogen bonding network of the former, shown by the shift to larger wavenumbers of the $-$OH stretching region of glycerol upon micellization. As g_0 is increased, the $-$OH stretching spectrum shifts to lower wavenumbers, indicating the presence of more hydrogen bonding and the formation of less bound or "free" glycerol, which is similar in trend to water incorporation into reverse micelles.[5,30] The large increase in droplet interactions at high g_0 shown in Figure 8(B) suggest the presence of "clusters" of reverse micelles which exist at conditions near phase inversion.[3,21] Temperature increases also lead to the onset of "clustering" or "percolation," which agrees with both the trend of Figure 8(B) showing the sharp $|\alpha|$ increase occurring at a lower g_0 for the 38°C case and the lowering of the phase boundary (i.e., the lessening of the amount of dispersed phase which can be solubilized) in Figure 1 for the higher temperature case. The reason(s) for the large increase in micellar size with g_0 shown in Figure 8(A) for cases near phase inversion are unknown, but may be due to deviations in shape from spheres. In terms of the rate of micellar solubilizate exchange, the presence of large quantities of glycerol causes no significant deviations from that encountered for water/AOT-based reverse micellar media.[9]

Figure 10 indicates a small quantity of fatty acid reduced the magnitude of micellar interactions significantly, which agrees with the trends of Figures 8(B) and 9(B), while additional fatty acid had little or no effect. This suggests fatty acid rigidifies the interface, meaning it increases interfacial curvature and decreases intermicellar attractions. In terms of mechanism, fatty acid increases the bending stress of the interface mainly by its incorporation into void space in the surfactant tail region. Indeed, a recent FTIR investigation[42] provides evidence of AOT/fatty acid interaction and hence the presence of fatty acid at the interface (fatty acid probably partitions between the micellar interface and the bulk organic continuum). In other words, the packing ratio, $v/a_0 l_s$, where v is the interfacial volume of the tail region; a_0 is the interfacial area per surfactant head group; and l_s is the length of the surfactant, increases upon addition of fatty acid by the increase in v, indicating a transformation toward rigidity. Thus, the net affect on micellar properties imposed by fatty acid is similar to that directed by long-tailed alcohols or high aqueous ionic strength.[27] In addition, Figure 1 indicates a lowering of the reverse micellar phase boundary with an increase in fatty acid concentration, which is consistent with the observation of decreased solubilization of dispersed phase by an increase in the interfacial rigidity.[27] (An exception is at very low w_0 values, where Fig. 1 demonstrates that fatty acid's presence increases glycerol solubilization. Here, an increase in rigidity is desirable to postpone "percolation" effects and phase inversion.) Our own FTIR experimentation[13] suggests fatty acid displaces glycerol (as well as water) from the vicinity of the surfactant head groups. This displacement may cause the apparent increase in R_h by the presence of fatty acid noted for Figure 8(A) for low g_0 values. The results of Figures 8(A), 9(A), and 10 indicate that fatty acid's presence in general creates little change in micellar size, except for media where "clustering" phenomena play a role [e.g., Fig. 8(A) for $g_0 \geq 4.0$].

Figure 9(B) demonstrates that the addition of water to glycerol-containing reverse micellar media lessens interdroplet attractions, but not to the same extent as fatty acid, suggesting water slightly increases interfacial rigidity. In terms of the packing ratio, the addition of water increases a_0 because the sulfonate groups of AOT are able to more fully ionize due to water's higher dielectric constant and therefore repulsion will exist between neighboring surfactant molecules. Along these same lines, FTIR data indicates water displaces glycerol away from the vicinity of the surfactant head groups.[13] However, to counteract the increase in a_0, the interfacial volume, v, will increase due to smaller micellar aggregation numbers and higher number concentrations of reverse micelles, moreover, due to an increase in overall surface area [Fig. 9(A) illustrates that R_h increases only slightly with w_0; thus, micellar aggregation numbers and number concentrations must decrease and increase, respectively]. Therefore, the packing ratio should increase slightly. The increase in v will allow for increased solubilization of fatty acid in water-containing reverse micellar media, a result which was obtained by us as noted in the previous section.

These results are useful in explanation of the kinetics. To obtain the highest yields, a significant amount of glycerol must be present at the interface. Thus, desirable operation would occur in media rich in glycerol content and lower in fatty acid and water, since the latter two species displace glycerol from the interfacial region. In agreement, conversions are low for w_0 values greater than 3.0 as shown on Figure 6(A). However, a significant amount of water is necessary for hydration of the enzyme's changed groups in order for it to function properly. In addition, small amounts of water may be beneficial by inducing an increase in a_0 and v, thus

allowing greater penetration and concentration of fatty acid at the interface. Along these same lines, Figure 1 illustrates that glycerol solubilization in the absence of fatty acid is increased by the addition of small quantities of water, which may be a consequence of the increased repulsion between neighboring surfactants. These reasons may help explain why conversions shown for Figure 6(A) at high g_0 and low w_0 values are small. In the "critical" region, conversions are highest: enough water is present to accommodate lipase and slightly "rigidify" the interface yet the water amount is not high enough to displace all of the interfacial glycerol. Indeed, Figure 8(B) demonstrates that intermicellar attractions are significantly high: $|\alpha| > 2.0$ for fatty acid-containing media at low g_0 values (i.e., in the "critical" region), which indicates the presence of glycerol in the vicinity of the interface. In agreement, the low values of g_0 correlates to the conditions of highest kinetic conversions at $w_0 = 1.5$ and 24°C.

Analysis of the relationship between initial fatty acid concentration and conversion shown in Figure 4 is more complicated. Recent kinetic results[13] indicate the large decrease in conversion at high initial fatty acid concentrations for media at high glycerol and low water content (e.g., the cases $w_0 = 2/g_0 = 3$ and $w_0 = 1/g_0 = 1.5$) is mainly due to rapid enzyme deactivation. The other cases of Figure 4 show a gradual drop in percent conversion as initial fatty acid concentration is increased, demonstrated by the decreasing of each curve's slope with $[\text{lauric acid}]_0$. This perhaps may be due to displacement of glycerol by fatty acid from the surfactant head region. Also a factor for producing the largest batch quantities of 1-monoglyceride is the increased solubilization of fatty acid in media containing significant amounts of water.

Of significant interest from our previous report[12] was the lessening of productivity for glycerol-fatty acid esterification for lipase from *R. delemar* in reverse micelles at temperatures higher than room temperature. Figure 7 gives strong evidence that the enzyme deactivates at 38°C, since the production of 1-monolaurin was dependent upon enzyme concentration and ceased to continue after a couple of hours, which is in stark contrast for the same experiment performed at 24°C. However, as mentioned previously, the enzyme itself has been shown to be quite active at high temperatures in the literature. In addition, we have found this enzyme (as well as lipase from *C. cylindracea*) to catalyze fatty-acid–butanol esterification in reverse micelles readily without significant activity loss at 38°C. Furthermore, two glycerol esterification reactions were conducted where the temperatures were altered in the middle of the reaction from 38 to 24°C to 24 and 38°C, respectively. In both cases, no further reaction occurred after the transition, indicating the enzyme irreversibly deactivated at the high temperature. Thus, high temperature must indirectly deactivate lipase from *R. delemar*. The exact temperature effect has not yet been deduced; but, the solubilization behavior of the enzyme may be a factor. To explain, at the higher temperature, the interface for media high in glycerol content will be quite fluid and disordered. This may allow lipase from *R. delemar* to partition away from the aqueous medium and become exposed to the bulk organic continuum, which would cause denaturation. This loss of activity by lipase from *R. delemar* towards glyceride synthesis must also be a function of the enzyme properties as well since lipase from *C. viscosum* is quite active towards this same reaction at 40°C.[7]

Does lipase-catalyzed esterification in reverse micelles stop due to kinetic equilibrium? We believe this is most often the case. For fatty acid-alcohol esterification/hydrolysis, two runs were performed: the first was the esterification of 100 m*M* of each *n*-butanol and lauric acid and the second was the hydrolysis of 100 m*M* lauric acid-butyl ester, with both containing similar reverse micellar media (e.g., at the same initial w_0) and operated under similar conditions. With the employment of either lipase from *R. delemar* or *C. cylindracea*, the final concentrations of fatty acid and ester for both reactions were identical ($[\text{lauric acid}]_{eq} = 10$ m*M*). These results also indicate the water concentration played an insignificant part in kinetic equilibria for this reaction, since the final water concentrations for each case were quite different. For glyceride synthesis, Figure 7 disqualifies enzyme deactivation for the reaction stoppage at 24°C. Fletcher et al.'s experimentation[7] also suggests equilibrium must be behind this reaction's stoppage. We have performed similar experiments as those mentioned by Fletcher et al., where additional substrate was added to the media during the reaction's course. Like these authors, the addition in many cases led to a new "equilibrium" condition. The results from these experiments disqualify reaction stoppage due to enzyme deactivation, since new equilibria were reached even for cases when substrate were added several days after the original equilibria were obtained. (However, when the same reactions were performed at 38°C, no further conversion took place, even when the additional substrate was added as early as one hour after the original reaction began.) These results will be published at a later date.[13] Furthermore, for the esterification reaction between the polyol ethylene glycol and fatty acid in reverse micelles, stoppage also appears to be linked to kinetic equilibrium since the final concentrations of substrates and products achieved are independent of lipase type.[14] However, again, severe enzyme deactivation causes premature reaction stoppage in media containing high amounts of glycerol and fatty acid with small amounts of water, as discussed above for Figure 4.

CONCLUSIONS

Glycerol–fatty-acid esterification with lipase from *R. delemar* produces mainly 1-monoglycerides at a maximum percent conversion of about 50–60% for fatty

acid. The highest productivity for this reaction appears to be related to the "critical" region of the phase diagram, which may in turn be related to high concentrations of substrates at the interfacial region, as indicated by light scattering. The reaction stoppage at room temperature appears to be linked to kinetic equilibrium in most cases, while at higher temperatures, enzyme deactivation is prevalent.

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