Enzymatic Glycerolysis of a Triglyceride in Aqueous and Nonaqueous Microemulsions

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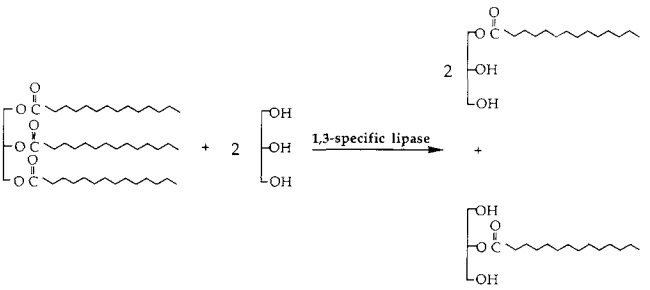
Enzymatic solvolysis of a model triglyceride, palm oil, was performed in microemulsions containing isooctane, sodium *bis*(2-ethylhexyl)sulfosuccinate (AOT), palm oil and a combination of water and glycerol as the polar component. Using a 1,3-specific lipase both hydrolysis, leading to the formation of fatty acid and one mole of monoglyceride, and glycerolysis, giving three moles of monoglyceride, occur. The reaction was very slow in a completely nonaqueous system. Addition of a small amount of water led to an increased rate of glycerolysis, in addition to hydrolysis. It was found that by using ^3H labelled material reaction products originating from the two reactions were formed in equimolar amounts. The products probably emanate from a common intermediate. The molar ratio, R, of water and glycerol to surfactant turned out to be critical, optimum R-value being 3.4. Four different lipases, one from porcine pancreas and three of fungal origin, were tested. No marked differences in ratio of monoglyceride to fatty acid formed were obtained, indicating that the ratio between glycerolysis and hydrolysis is constant regardless of the lipase used.

The use of microemulsions of low water content as media for enzymatic reactions has been the topic of several recent papers from our group (1-3), as well as from others (4-9). The majority of work has been performed with lipases and it has been demonstrated that the enzyme retains a high intrinsic activity in these highly nonpolar solutions. Immobilized enzymes can also be used in a microemulsion medium without too much impairment of reaction kinetics (10). The double-tailed anionic surfactant sodium *bis*(2-ethylhexyl)sulfosuccinate (AOT) has been most widely used but microemulsions based on nonionic, as well as cationic, surfactants have also been employed.

The microemulsions used have been characterized by various methods, such as pulsed-gradient spin-echo NMR (3) and dynamic light scattering (7). The systems based on ionic surfactants have all been found to consist of discrete droplets of water in oil. The situation is less clear for the systems based on nonionics; those having a water content of at least a few percent also seem to consist of water droplets in oil continuum (3) whereas those having very low water content probably do not exhibit the reverse micelle structure (1,11).

One enzymatic reaction in microemulsion media that has been investigated in some detail is the selective hydrolysis of trigylceride to 2-monoglyceride using a 1,3-specific lipase (2,3). It has been found that the reaction runs smoothly in microemulsions based on AOT. At optimum conditions the yield of monoglyceride is around 75%, unreacted triglyceride and glycerol accounting for most of the balance. Of course, each mole of monoglyceride formed is accompanied by the release of two moles of fatty acid.

Relatively recently it has been demonstrated that glycerol can replace water as polar component of at least some microemulsion-type systems (12-14), and such systems have been used for lipase catalyzed formation of oleate esters of glycerol (15). Substituting water for glycerol in microemulsions for enzymatic solvolysis of triglyceride would be of considerable interest because it opens the possibility to perform selective glycerolysis of the triglyceride instead of hydrolysis. A regioselective glycerolysis would lead to the formation of two moles of 1-monoglyceride and one mole of 2-monoglyceride, as is shown in Scheme 1. The main advantage with this process compared with the corresponding hydrolysis is that monoglyceride is formed without concomitant liberation of fatty acid.



SCHEME 1. Enzymatic glycerolysis of a triglyceride.

It is the aim of this present work to study the use of glycerol containing microemulsions for enzymatic solvolysis of a model triglyceride, palm oil.

EXPERIMENTAL

Materials. Glycerol (Sigma grade) was purchased from Sigma Chemical Co. (St. Louis, MO), and palm oil from Aarhus Olie Fabrik, Denmark. The anionic surfactant sodium *bis*(2-ethylhexyl)sulfosuccinate was supplied by Cyanamide (Wayne, N.J.) and used without further purification. The lipases used were obtained from Serva, Germany (*Rhizopus sp.*, 65-70 U/mg), Calbiochem, USA (*Rhizopus arrhizus*, 4509 U/mg), Fluka, Switzerland (*Rhizopus delemar*, 63.1 U/mg) and Sigma (Porcine pancreas). Radioactive labelled [1(3)^3H] glycerol was purchased from Amersham, Great Britain. All other chemicals were reagent grade.

Methods. The enzymatic reactions were carried out at 37°C with magnetic stirring in 100 ml bottles. The reaction mixtures consisted of isooctane:palm oil at a constant weight ratio of 20:1, AOT, glycerol and buffered water solutions at pH 7.0. In all experiments 2000 units of lipase were used.

After completing the reaction the enzyme was denatured by heating the mixture at 100°C for 10 minutes. The

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solutions were analyzed for monoglycerides by a method proposed previously (16) using a Varian 3400 gas chromatograph with a fused silica column (5 m, 0.22 mm i.d., 0.05 μm OV 1) with commercial monooleate and tricaprin as internal standards.

Monoglycerides were also detected using ^{3}H labelled glycerol which was mixed with inactive glycerol and employed as starting material in the same pattern as previously. After completing the reaction, the monoglycerides were isolated by preparative thin layer chromatography. The thin layer plates were eluated with hexane, diethyl ether, acetone (60:30:10 by volume) and visualized with iodine. The monoglyceride fraction was scraped off the plate and extracted with diethyl ether. The ether solution was evaporated and the monoglycerides analyzed in a liquid scintillation counter for radioactivity. A standard curve was prepared from the radioactive glycerol used as starting material, and the amount of radioactive monoglyceride formed during the reaction was calculated.

Analysis for fatty acid was made by titration with 0.1 M KOH in ethanol using a sample without lipase as a blank. In all experiments molecular sieve-dried (3Å) isooctane was employed. The water content in isooctane was analyzed to be less than 0.1% by volume using the titrimetric method of Karl Fischer. Percent values given are weight percent unless otherwise stated.

RESULTS

Phase diagram. The pseudo-ternary phase diagram for the system oil, AOT and glycerol at 37°C was constructed. Palm oil:isooctane at a constant weight ratio of 1:20 was used as the oil component, and the oil-rich corner of the diagram is shown (Fig. 1). Phase diagrams for four different water concentrations were constructed. Successive additions of glycerol were made to the system and the effect of increasing water and glycerol content on phase behavior is shown in the diagram. As can be seen, incorporation of only minute amounts of water considerably enlarges the isotropic one-phase domain, the L2 phase, denoted I, and reduces the two-phase area, II. It is important to note, however, that also in the nonaqueous system at least 1% glycerol can be incorporated into all isooctane:AOT combinations. All enzymatic experiments were made at compositions lying in the L2 phase.

Effect of water content. Figure 2 shows the formation of monoglyceride and fatty acid as a function of water content. As can be seen, reaction in entirely water-free medium gives a very low degree of conversion of triglyceride. Increasing amounts of water in the system result in an increase in the amount of fatty acid formed, as expected. Monoglyceride formation, on the other hand, seems to go through a maximum around 0.5% water in the system. This value corresponds to a molar ratio of water to surfactant, R_w, of 2.5 and to a molar ratio of water + glycerol to surfactant, R_{w+g}, of 3.4.

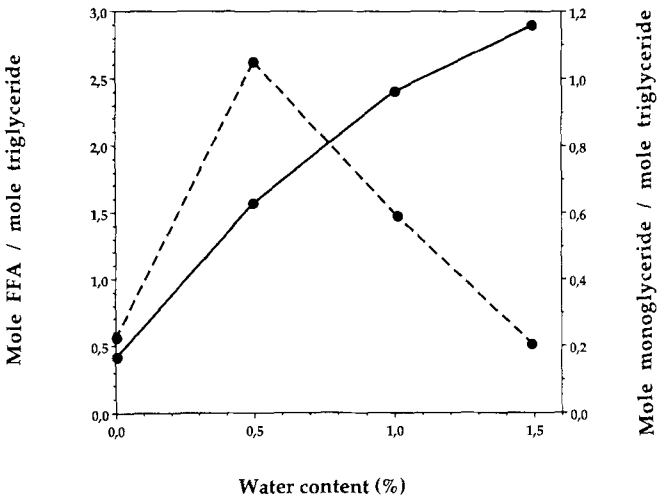


FIG. 2. Effect of water content on the formation of monoglyceride (- - -) and fatty acid (——) from palm oil. Reactions were run at 37°C for 72 hr. Glycerol and AOT contents were 1 and 5%, respectively.

Effect of surfactant content. The formation of monoglyceride and fatty acid as a function of AOT concentration is shown in Figure 3. As can be seen, the amount of

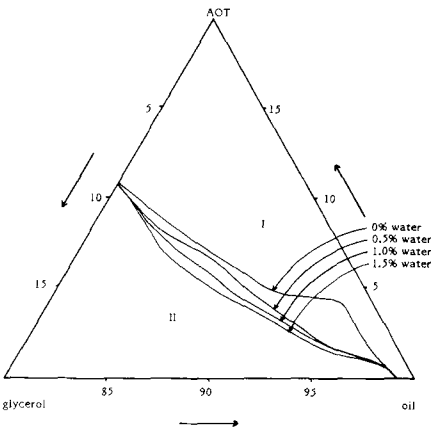


FIG. 1. Oil-rich corner of phase diagram at 37°C for the system oil, AOT and glycerol with varying amounts of water added. The oil component consists of isooctane: palm oil in a weight ratio of 20:1. (I) Indicates isotropic phase and (II) two-phase system.

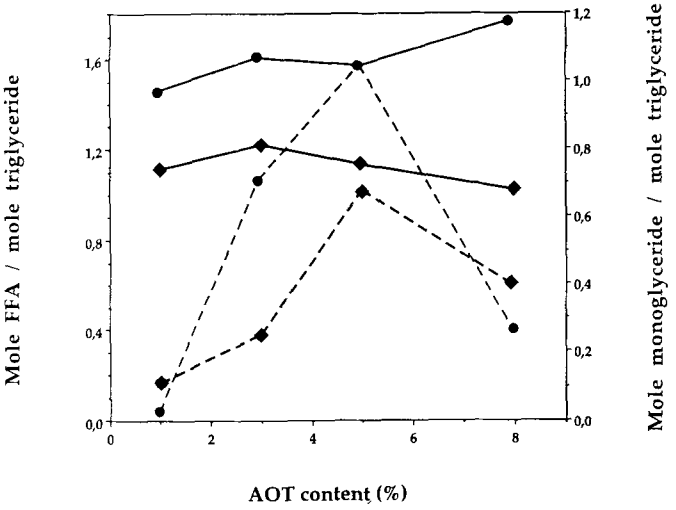


FIG. 3. Effect of AOT content on the formation of monoglyceride (- - -) and fatty acid (——) from palm oil. Reactions were run at 37°C for 6 (◆) and (●) 72 hr. Glycerol and water contents were 1 and 0.5%, respectively.

momoglyceride formed goes through a maximum at around five weight percent surfactant, both at 6 hr and at 72 hr reaction time. This corresponds to the same maxima of R-values as given above. The amount of fatty acid formed seems to be relatively independent of the concentration of AOT.

Choice of lipase. Figures 4 and 5 show the effect of the source of the enzyme on the reaction. Two observations can be made from the graphs: The pancreatic lipase is considerably less active than the microbial lipases, a fact which may be attributed to a colipase dependence of the former enzyme (17). It has been claimed, however, that the presence of colipase is required only in systems containing high concentrations of bile salts (18). And, there are no marked differences in ratio of monoglyceride to fatty acid formed, indicating that the ratio between glycerolysis and hydrolysis is constant regardless of the lipase used.

Effect of glycerol content. The effect of the glycerol content on monoglyceride and fatty acid formation at two different concentrations of water is illustrated in Figure 6. The yield of fatty acid decreases as the glycerol content is raised, the effect being more pronounced at the lower water content. The formation of monoglyceride increases with the glycerol concentration and reaches a plateau value at about 4% glycerol. This value is approximately constant--1.2-1.4 mole monoglyceride per mole starting triglyceride--regardless of the water content. As expected, the most favorable ratio between monoglyceride and fatty acid is obtained at high glycerol and low water content. As can be seen in the phase diagram (Fig. 1), the glycerol content cannot be raised above 8 and 9% at an AOT concentration of 8% and water contents of 0.5 and 1.0%, respectively. The decrease in monoglyceride formation at high glycerol content and 0.5% water may be interpreted as being due to diglyceride formation.

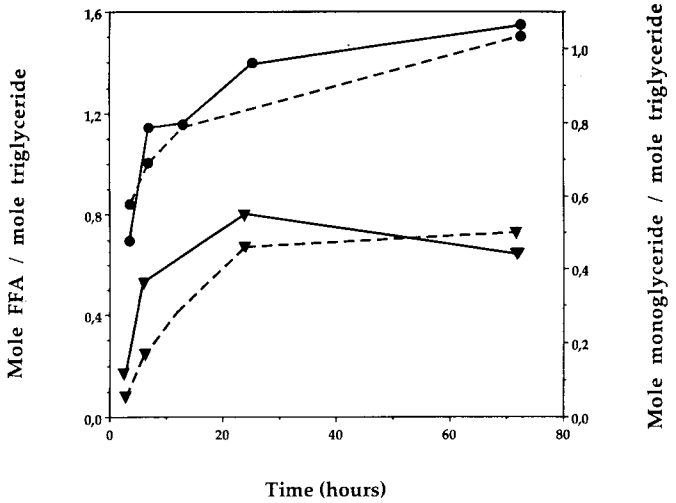


FIG. 4. Formation of monoglyceride (- - -) and fatty acid (——) from palm oil with lipase from porcine pancreas (▼) and *Rhizopus sp.* (●). Reactions were run at 37°C for 24 h. The glycerol, water and AOT contents were 1, 0.5 and 5%, respectively.

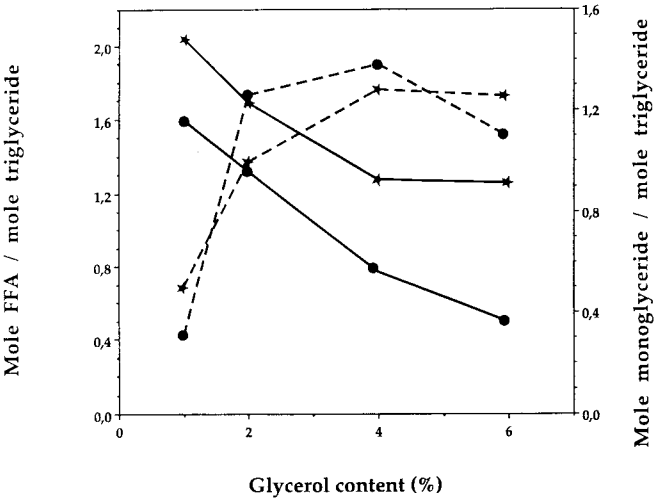


FIG. 6. Effect of glycerol content on the formation of monoglyceride (- - -) and fatty acid (——) from palm oil. Reactions were run at 37°C for 24 h. The AOT concentration was 8% and the water content 0.5 (●) and 1.0 (∗)%.

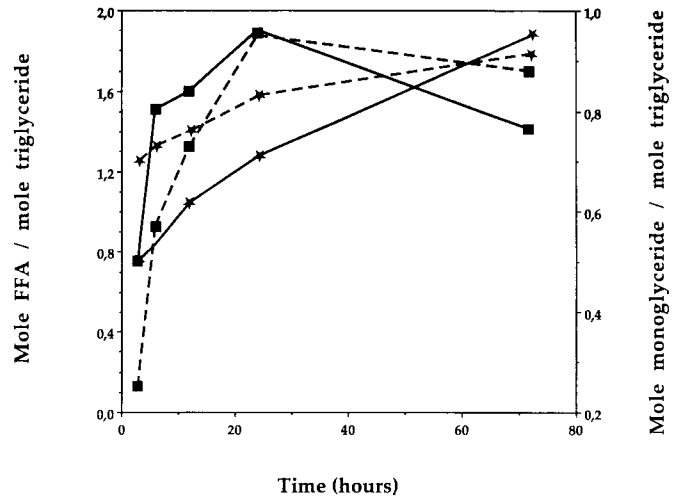


FIG. 5. Formation of monoglyceride (- - -) and fatty acid (——) from palm oil with lipase from *Rhizopus delemar* (∗) and *Rhizopus arrhizus.* (■). Reactions were run at 37°C for 24 h. The glycerol, water and AOT contents were 1, 0.5 and 5%, respectively.

Reaction with labelled glycerol. Reaction with 0.5% water and 2.6% glycerol, corresponding to equal concentrations of the two solvents, was carried out with ^{3}H labelled material. The formation of radioactive monoglyceride was monitored during 48 hr reaction time. The result is shown in Figure 7. As can be seen from the graph, the ratio of labelled to unlabelled product is approximately constant around 1 throughout the reaction period.

DISCUSSION

As shown in Figure 1, an isotropic single phase is formed in the "oil corner" of the system. In the absence of water this L2 phase is small, and only minor amounts of glycerol can be incorporated into the system before phase separation occurs. In such completely nonaqueous systems the enzymatic reaction is extremely sluggish, as had been observed earlier. Evidently, some water has to be added in order to obtain enzymatic activity.

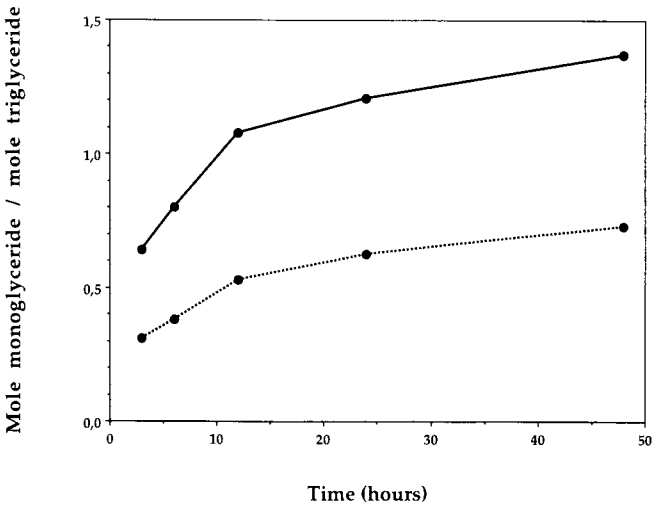
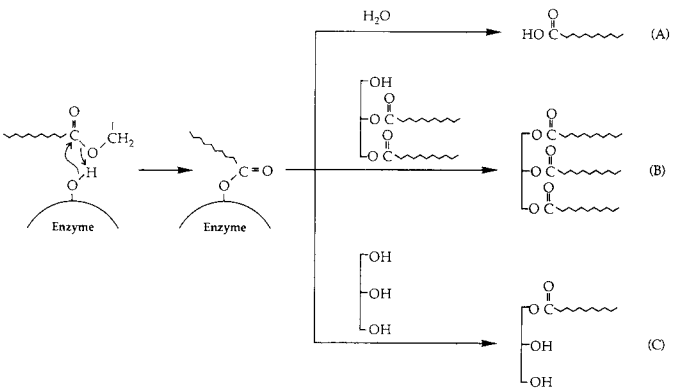


FIG. 7. Formation of radioactive labelled (- - -) and unlabelled (——) monoglyceride from palm oil. Reactions were run at 37°C with 0.5% water and 2.6% glycerol (corresponding to equal concentrations). The AOT content was 5%.



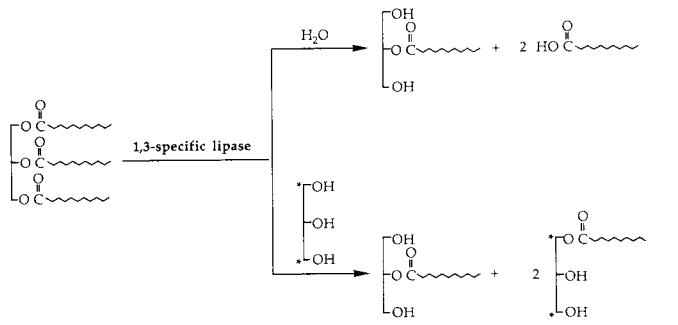
SCHEME 2. Schematic illustration of the three different routes of reaction originating from the enzyme-substrate complex. Reaction route (A) demonstrates glycerolysis, (B) alcoholysis with diglyceride and (C) glycerolysis.

Addition of a small amount of water leads to an increase of the area of the L2 phase in the phase diagram and to improved enzymatic activity. Maximum yield of monoglyceride is obtained at a water content of 0.5%. The molar ratio of polar component to surfactant, the R-value, has previously been found to be of importance for reaction kinetics in other systems since it governs the size of droplets (3,7). In this work, variations of both water content at fixed surfactant concentration (Fig. 2) and surfactant content at fixed water concentration (Fig. 3) lead to the same optimum in R-value, 3.4. For an AOT-based microemulsion with glycerol as polar component, this R-value corresponds to a droplet size of *ca* 4.7 nm (12).

The active site of lipases is relatively nonspecific in that it hydrolyzes tri-, di- and monoglycerides, as well as phospholipids. However, the positional specificity of hydrolysis for 1,3-specific lipases is strict for all substrates with a preferential cleavage of the *sn*-1(3) ester bonds with the subsequent slow isomerization of the 2-monoglyceride to 1-monglyceride and total hydrolysis. Although little direct evidence exists for the involvement of an active site serine residue in the action of the enzyme, a "serine esterase-like" mechanism is best able to accommodate observations from covalent modification experiments on lipases (19). Enzyme-substrate formation is believed to involve hydrogen bonding to C-O-C oxygens of the substrate (20). The carbonyl carbon is then subject to a nucleophilic attack by the serine hydroxyl group (or by some other nucleophile). Under normal aqueous conditions the serine ester formed is subsequently hydrolyzed. If the water activity of the system is low, as in reverse micelle systems, the serine ester may be split through alcoholysis, e.g., by a mono- or diglyceride. This is the mechanism for enzymatic transesterification. If water is replaced by glycerol as polar component of the system, the intermediate serine ester may undergo glycerolysis instead of hydrolysis, or alcoholysis by mono- or diglyceride. The various reaction routes are illustrated in Scheme 2.

Increasing the ratio of glycerol to water leads to increased glycerolysis and decreased hydrolysis. This effect is clearly seen in Figure 6. Assuming absolute regiospecificity of the enzyme and no acyl migration, hydrolysis alone will lead to two moles of fatty acid and one mole of monoglyceride per mole starting triglyceride. Complete glycerolysis will give three moles of monglyceride per mole triglyceride. At equal concentrations of water and glycerol in the system the ratio of fatty acid to monglyceride should be indicative of the relative rates of hydrolysis and glycerolysis of the intermediate enzyme ester, equal rate constants corresponding to a 1:2 ratio. As can be seen in Figure 6, the molar ratio of fatty acid to monoglyceride at equal concentrations of water and glycerol (e.g., 0.5% water-2.6% glycerol or 1.0% water-5.1% glycerol) is close to 1:1, indicating a preference for hydrolysis. However, acyl group migration from 2- to 1-monoglyceride is known to occur in related systems, although at a slow rate (3). Migration followed by hydrolysis will increase the fatty acid to monoglyceride ratio. Consequently, unless the extent of migration is known, the relative rates of hydrolysis and glycerolysis cannot be calculated from the ratio of fatty acid to monoglyceride formed.

The experiments with labelled glycerol reveal the source of the glycerol moiety of the monoglyceride



SCHEME 3. Enzymatic hydrolysis and glycerolysis using ^3H labelled glycerol.

formed. Whereas hydrolysis gives only unlabelled monoglyceride, glycerolysis leads to the formation to two moles of labelled and one mole of unlabelled monoglyceride per mole of triglyceride, as shown in Scheme 3. Figure 7 shows that, at equal concentrations of glycerol and water in the microemulsion, the amount of labelled monoglyceride is about half of the total amount throughout the reaction period. This indicates that the rate of glycerolysis approximates that of hydrolysis. Continued hydrolysis to glycerol and fatty acid will only give a marginal effect on the glycerol to water ratio because the solvents are used in large excess over the triglyceride.

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