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Effects of Emulsifier Charge and Concentration on Pancreatic Lipolysis. 1. In the Absence of Bile Salts

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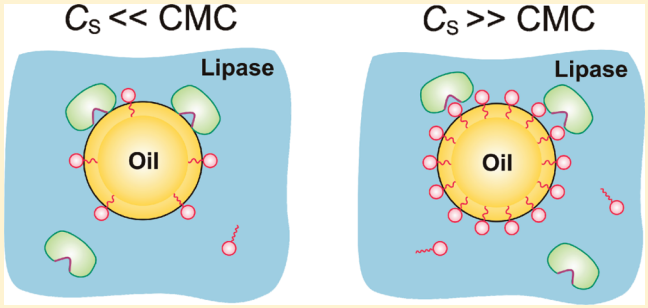
Supporting Information

ABSTRACT: An in vitro study is performed with sunflower oil-in-water emulsions to clarify the effects of type of used emulsifier, its concentration, and reaction time on the degree of oil lipolysis, α. Anionic, nonionic, and cationic surfactants are studied as emulsifiers. For all systems, three regions are observed when surfactant concentration is scaled with the critical micelle concentration, C_S/cmc: (1) At C_S < cmc, $\alpha \approx$ 0.5 after 30 min and increases up to 0.9 after 4 h. (2) At $C_S \approx 3 \times$ cmc, $\alpha \approx$ 0.15 after 30 min and increases steeply up to 0.9 after 2 h for the cationic and nonionic surfactants, whereas it remains around 0.2 for the anionic surfactants. (3) At C_S above certain threshold value, α = 0 for all studied surfactants, for reaction time up to 8 h. Additional experiments show that the lipase hydrolyzes molecularly soluble substrate (tributirin) at $C_S \gg$ cmc, which is a proof that these surfactants do not denature or block the enzyme active center. Thus, we conclude that the mechanism of enzyme inhibition by these surfactants is the formation of a dense adsorption layer on an oil drop surface, which displaces the lipase from direct contact with the triglycerides.



1. INTRODUCTION

Triglycerides (TG) are the main constituents of vegetable and animal fats and are the nutrients with the highest energetic value. Metabolic deviations related to fat digestion may lead to obesity and to a range of related health problems, such as cardiovascular diseases and diabetes mellitus type 2.[1−3] Therefore, the studies aimed to reveal the main factors controlling fat lipolysis are of major interest in several contexts—weight management, health diets, prevention of cardiovascular diseases, etc.

TG lipolysis occurs in the gastrointestinal tract (GIT), mainly in the small intestine, under the action of pancreatic lipase.[4] The pancreatic lipase (PL) catalyzes the lipolysis at sn-1 and sn-3 positions of the TG, leading to formation of monoglycerides (MG) and free fatty acids (FFA). PL operates at around the neutral pH and has to be adsorbed on the oil−water interface in order to come in direct contact with the water-insoluble TGs and to catalyze the lipolysis reaction.[4,5] A number of endogenous surface-active molecules, such as bile salts and phospholipids, and some of the reaction products (FFA, MG) are known to coadsorb on the TG−water interface.[6] Under physiological conditions, these endogenous surfactants do not hinder the fat lipolysis. Furthermore, these biosurfactants form molecular aggregates (dietary micelles) that solubilize the lipolysis products in the aqueous phase and thus ensure their transport across the mucus layer of the intestinal walls.[7,8] One should note the important role of the pancreatic colipase (cofactor of about 10 kDa),[4] which enables lipase to overcome the inhibition by bile salts; see, for example, the review by Lowe.[4]

In the human diet, the TG are regularly ingested in the form of food emulsions, such as milk and various dairy products, mayonnaise, ice cream, etc.[9] These emulsions are stabilized by emulsifiers that also adsorb on the oil−water interface and, thus, could affect the adsorption of the natural components of the GIT juice (lipase, bile salts, fatty acids) and the entire lipolysis process. This competitive adsorption of the various components has been considered in the literature as one possible way for weight management, via delayed TG lipolysis.[10] Physiologically, the TG lipolysis and most of the absorption of its products are completed in the duodenum and jejunum.[11,12] If fatty acids reach the ileum, they activate the so-called "ileal brake" mechanism, where their presence stimulates the secretion of satiety-inducing hormones from the intestinal cells.[13] This ileal brake mechanism is believed to trigger an increase in the sense of satiety at lower energy intake.[14,15]

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For these reasons, the effect of food-grade emulsifiers on the kinetics of fat digestion has been actively studied in recent years.[16−24] A review by Reis et al.[16] describes the ability of sn-2 MG to inhibit the lipase action on a tricaprylin substrate in the so-called "TIM" in vitro system. The proposed mechanism of inhibition is that sn-2 MG takes over the interface and displaces tricaprylin and lipase molecules from the interface. The latter conclusion was supported by calculations of the interfacial coverage by sn-2 MG molecules at the phosphate buffer/decane interface.[16]

Chu et al.[17] reported the inhibition of pancreatic lipase by digalactosyldiacylglycerol (DGDG), observed in vitro. Model experiments showed that the layer of DGDG, adsorbed at the air−water interface, is more resistant to adsorption of bile salts, lipase, and colipase, compared to the control layer of dipalmitoylphosphatidylcholine (DPPC).[25]

The inhibition of pancreatic lipase by basic biopolymers was reviewed by Tsujita.[18] The author evaluated the effect of polymer concentration on the lipase activity in the presence and in the absence of different surfactants. The mechanism of inhibition is shown to be the inability of lipase to adsorb at the TG−water interface, because the biopolymer makes a relatively dense adsorption layer. In vivo studies on mice confirmed the antiobesity effect of ε-polylysine as one example of this class of biopolymers.[26]

Gargouri et al.[19] reports the inhibition of pancreatic lipase by various surfactants in the absence of bile salts and subsequent lipase reactivation after addition of bile salts. The effect of surfactant and bile salts concentrations on lipase activity was studied. However, no clear conclusion about the mechanisms of lipase inhibition by surfactants and the lipase reactivation by bile salts was drawn in this study.

Recently, Li et al.[20] studied the effect of low molecular weight surfactants on the lipase activity under simulated gastrointestinal conditions. It was found that, at sufficiently high concentrations, all studied surfactants are able to inhibit the pancreatic lipase. To explain this effect of the surfactants, the authors suggested several possible mechanisms, without trying to define which of them is operative.

The aim of the current study is to investigate systematically the effect of low molecular mass surfactants (as emulsifiers) on the TG lipolysis by pancreatic lipase and to provide a mechanistic explanation of the observed effects. The studied factors are the surfactant concentration, charge, and length of the hydrophobic tail (varied between 12 and 16 carbon atoms). On purpose, to clarify the effect of surfactant type, we have chosen a series of surfactants that are not necessarily of food grade; in this way we could focus on the physicochemical factors involved in the process of lipase inhibition by surfactants and in the related process of lipase reactivation by bile salts.

In the current first part of this series of papers, we study the effect of emulsifiers in the absence of bile salts. In section 2 of the current paper we describe the materials and methods used, in section 3 we present the experimental results and discuss the mechanism of pancreatic lipase inhibition by the studied surfactants, and in section 4 we summarize the conclusions. In the subsequent paper of this series,[27] we study the interactions between the same emulsifiers and bile salts, in the context of lipase reactivation by the bile salts.

2. MATERIALS AND METHODS

2.1. Materials. As triglyceride we used commercial sunflower oil (SFO) which was purified from polar contaminants by multiple passes through a chromatography column, filled with Florisil adsorbent, until the interfacial tension with pure water became ≈32 mN/m.[28] In one series of experiments, we used tributyrin (Sigma-Aldrich, 99% purity), which was used as received.

In the thin-layer chromatography, we used 2-oleoylglycerol as a MG standard (product of Sigma, Cat N M2787) and 1,2-dioleoyl-*rac*-glycerol as a DG standard (product of Sigma, Cat N D8394), as well as oleic acid (OA, product of Tokyo Kasei Kogyo) and stearic acid (product of Merck, Cat N 800673) as fatty acid standards. As standard for triglycerides we used triolein (product of Sigma, Cat N T7140). As internal standards we used also cis-unsaturated monoglycerides of technical food grade (product of Danisco).

Pancreatin from porcine pancreas (Sigma-Aldrich, Cat N P8096), containing a range of enzymes including amylase, trypsin, lipase, ribonuclease, and protease, was used as source of pancreatic lipase and colipase (at 1:1 molar ratio in the pancreatic source[29,30]).

Several low molecular mass surfactants were compared as emulsifiers to vary both the surfactant head groups and hydrophobic tails: nonionic surfactants polyoxyethylene-20 sorbitan monolaurate (Tween 20, product of Fisher, molecular mass M = 1227 g/mol) and polyoxyethylene-20 sorbitan monooleate (Tween 80, product of Fluka, M = 1310 g/mol), anionic surfactants sodium lauryl ether sulfate (SLES, product of Stepan, M = 332 g/mol) and α-olefin sulfonate (AOS, product of Teokom, M = 350 g/mol), and cationic surfactants dodecyltrimethylammonium bromide (DTAB, product of Sigma, M = 308 g/mol) and cetyltrimethylammonium bromide (CTAB, product of Sigma, M = 364 g/mol). All emulsifiers were used as received.

All solutions were prepared with deionized water, which was purified by an Elix 3 water purification system (Millipore). As buffer we used Tris (product of Merck, M = 121 g/mol). For adjustment of pH we used HCl (Sigma) and NaOH (Teokom). For preparation of salt solutions we used NaCl and KCl (products of Merck) and $CaCl_2$ (product of Fluka).

2.2. Emulsion Preparation. Oil-in-water emulsions were prepared by mixing surfactant solution with sunflower oil, at an oil volume fraction of 30, 60, 70, or 80 vol %. The total volume of the emulsions was 50 mL. The emulsifier solution contained 1 wt % emulsifier, 10 mM NaCl, and 0.01 wt % NaN_3. Rotor-stator homogenizer Ultra Turrax T25 (Janke & Kunkel GmbH & Co, IKA-Labortechnik), operating for 5 min at 13 500 rpm, was used for emulsification. The formed emulsions were stored in glass jars, at room temperature, for no more than 1 week. Before usage, these emulsions were rehomogenized by a gentle hand-shaking. The necessary amount of emulsion was taken by a pipet.

2.3. Emulsion Drop Size Determination. The drop size distribution in the obtained emulsions was determined by video-enhanced optical microscopy.[31,32] The oil drops were observed and recorded in transmitted light with an Axioplan microscope (Zeiss, Germany), equipped with an Epiplan ×50 objective and connected to a CCD camera and video recorder. The diameters of the recorded oil drops were measured one-by-one by an experienced operator, using a custom-made image analysis software. For each sample, the diameters of at least 3000 drops were measured. The accuracy of the optical measurements was estimated to be ±0.3 μm.[31] The mean volume-surface diameter

$$d_{32} = \sum N_i d_i^3 / \sum N_i d_i^2 \quad (1)$$

was used as a characteristic of the mean drop size in the studied emulsions (here N_i is the number of measured drops with diameter d_i).

2.4. In Vitro Lipolysis Model. First, we prepared the following stock solutions, which were mixed afterward to prepare the final solutions for the actual lipolysis experiments: (a) Saline solution containing 150 mM NaCl + 5 mM KCl, (b) electrolyte solution containing 5 mM Tris + 15 mM $CaCl_2$ + 40 mM NaCl, and (c) pancreatic solution containing 30 mg pancreatin + 5 mL of saline solution (corresponding to 0.6 wt % pancreatin + 150 mM NaCl + 5 mM KCl). Saline and electrolyte solutions were prepared and stored at room temperature. Before mixing these solutions with the pancreatic solution, they were thermostated at T = 37 °C. The pancreatic

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solution was prepared directly at 37 °C, just before its use in the actual experiments. In the experiments aimed at determining the effect of emulsifier concentration on the enzyme activity, the required amount of emulsifier was dissolved in the saline solution.

The order of solution mixing is shown schematically in Figure 1. First, the appropriate amount of emulsion was added to 17.5 mL saline

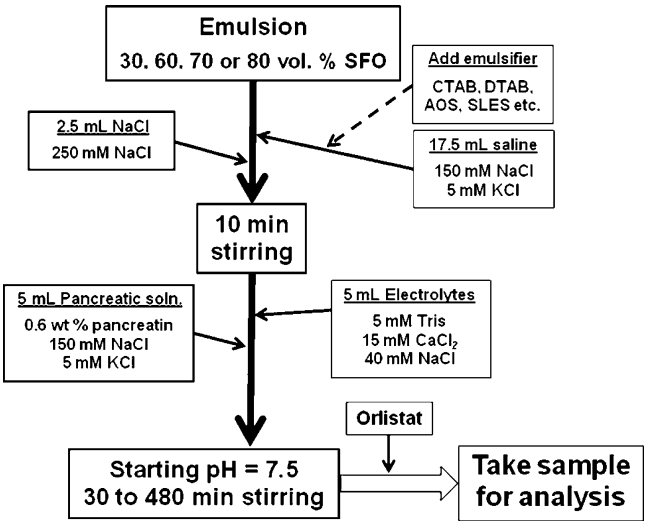

Figure 1. Schematic presentation of the used in vitro procedure (no bile acids are present in the reaction mixture).

solution by a micropipet. The amount of added emulsion was calculated to give always a final concentration of 0.2 wt % sunflower oil, independent of the oil volume fraction of used emulsion (217 μL from 30 vol % emulsion, 109 μL from 60 vol % emulsion, 93 μL from 70 vol % emulsion, and 82 μL from 80 vol % emulsion). Second, 2.5 mL of 250 mM NaCl solution was added and the mixture was stirred for 10 min. Afterward, 5 mL of electrolyte solution was added and pH was adjusted to 7.5 with small aliquots of HCl and NaOH. Finally, we added pancreatic solution and stirred for the required reaction time. The final composition of the reaction mixture is 0.2 wt % SFO, 0.1 wt % pancreatin, 0.83 mM Tris, 2.5 mM $CaCl_2$, 140 mM NaCl, and 3.75 mM KCl. To stop the lipolysis at the end of the experiment, we added 24 mg of Xenical granules (Roche), which contain the pancreatic lipase inhibitor tetrahydrolipstatin (Orlistat).[33]

This in vitro procedure is designed to study the effect of emulsifiers in the absence of bile salts. In the second paper of this series,[27] a modified procedure is applied that involves bile salts, in order to study the combined effect of emulsifiers and bile salts on TG lipolysis.

2.5. Experiments at Fixed pH. In the procedure described in section 2.4, the pH of the reaction mixture decreased with the reaction time, due to the formation of fatty acids in the course of lipolysis. To check for the effect of pH on TG transformation, we applied the same in vitro lipolysis model, with the only difference being that pH = 7.5 was maintained constant using the pH-STAT apparatus (Titrando 842, Metrohm). In this way, the released free fatty acids were automatically titrated with 25 mM NaOH to maintain constant the solution pH. The titrated amount of NaOH was not used to determine the degree of TG hydrolysis because we found that not all released free fatty acids are titrated; a fraction of the free fatty acids remains dissolved in the oily drops (for further explanations, see section3.2 below). Therefore, the degree of TG transformation was determined using TLC as in all other experiments (except for those involving tributyrin lipolysis; see Figure 8).

For determination of the pancreatic lipase activity on a molecularly water-soluble substrate, we used tributyrin with a final concentration in the reaction mixture of 0.0058 wt %. The tributyrin was dissolved in the saline solution at a concentration of 0.01 wt %, by stirring for 24 h at T = 37 °C. A separate solution of saline, not containing tributyrin, was used to prepare the pancreatic solution. The butyric acid released by the hydrolysis of tributyrin is highly soluble in water, and therefore, the degree of lipolysis reaction was determined for this substrate by titration with NaOH. The NaOH was standardized with potassium hydrogen phthalate (product of Teokom).

2.6. Procedure for Extraction of the Nonhydrolyzed TG and Reaction Products by Chloroform for TLC Analysis. After terminating the lipolysis reaction by the addition of Orlistat, we let the reaction mixture cool down to room temperature and decreased its pH down to pH = 2 by adding HCl (to decrease the solubility of the reaction products in the aqueous phase). Next, 6 mL of chloroform was added and the sample was sonicated for 15 min. After every 5 min of sonication, the sample was agitated by vigorous hand shaking. The obtained complex dispersion was centrifuged for 30 min at 4500 rpm, which led to separation of the aqueous and organic phases. Both phases were clear after the centrifugation, indicating that the oily drops were entirely transferred into the chloroform phase. The obtained chloroform phase was further analyzed by TLC.

2.7. Thin Layer Chromatography (TLC). The degree of TG lipolysis was determined by analysis of the reaction mixture using TLC. We used aluminum-backed silica gel 60 F 254 plates, with dimensions 20 × 20 cm^2. The start line was drawn at 2 cm from the bottom of the plate, using a soft pencil. The start-front distance was 15 cm. The samples were deposited on the plate with 5 μL capillaries, at 1.5 cm distance from each other. As carrier liquid phases we used the following organic mixture: petroleum ether (product of Merck, Cat N 1.01775.5000), diethyl ether (product of Merck, Cat N 1.00921.100), and acetic acid (product of Teokom) at the ratio 80:20:1 by volume. The chromatography chamber was presaturated with vapors of the carrier liquid (for at least 15 min) before introducing the TLC plate into it. The depth of the carrier liquid in the chamber was always below 1.5 cm. After the carrier liquid had reached the front line, the plate was taken out of the chamber and allowed to dry for 15 min. The plate was then dipped for 1−2 s in a 6 wt % phosphomolybdic acid (product of Riedel de Haen, Cat N 31426) dissolved in 2-propanol, dried again for 15 min, and heated to 100 °C for 15 min to visualize the spots of the separated components. During heating, the molybdic acid reacts with the lipids, forming "molybdenum blue" spots, which visualize the various lipid components.[34]

This procedure allowed us to observe spots corresponding to triglycerides (TG), diglycerides (DG), monoglycerides (MG), and fatty acids (FFA); see Figure 2. The positions of the various types of lipids were identified with reference substances, as described in section 2.1.

2.8. Quantitative Determination of the Triglycerides and Lipolysis Products in the Reaction Mixture. The TLC chromatograms, obtained as described in section 2.7, were scanned on an HP Scanjet G2410 scanner (Hewlett-Packard) with a resolution of 600 dpi. The scanned image was converted into grayscale and inverted with the Corel Photo Paint 12 package, so that the spots appear bright on a dark background. The exported TIFF-file with the inverted image was imported into custom-made software for image analysis, which allows one to determine the intensity, I, as a function of the vertical position, y, in a rectangle covering the analyzed lane in the chromatogram. More precisely, the software obtains the mean intensity, as a function of the vertical position, in each lane of the chromatogram.

To determine the peak areas, we fitted each of the peaks by a Gaussian curve with four free parameters

$$I = I_B + A \exp\left[-\frac{(y - y_0)^2}{2B^2}\right] \quad (2)$$

where I_B is the intensity of the background (baseline), A is a constant proportional to the peak height, y_0 is the position of the peak maximum, and B is the peak width.

After performing the numerical fit and determining the parameters involved, we used the values of A and B to calculate the peak area. To determine the concentration of the substance producing this peak, we prepared calibration curves with solutions of known concentrations. We used standard solutions of SFO and oleic acid in the concentration range from 0.01 to 1 wt % (these concentrations are given with respect

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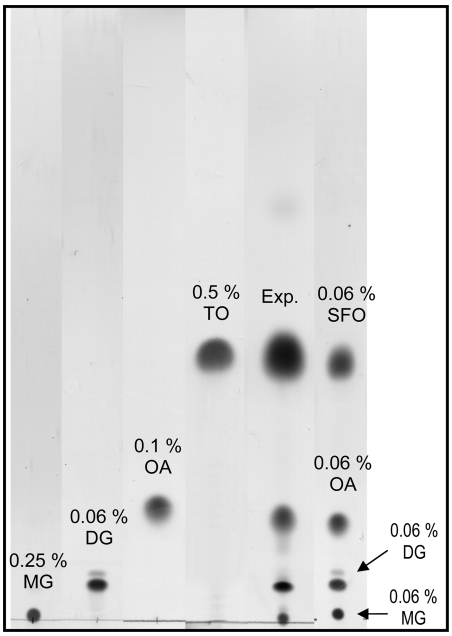

Figure 2. Representative TLC images of the reference substances, used for identification of the reaction products on the chromatogram. The carrier phase is petroleum ether:diethyl ether:acetic acid at 80:20:1 ratio by volume. The label "Exp." (viz., experiment) refers to SFO stirred in pancreatic solution for 30 min at 37 °C. Abbreviations: TO = trioleine, SFO = sunflower oil; OA = oleic acid; MG = monoglyceride; DG = diglyceride.

to the chloroform extract used for the TLC analysis). The obtained calibration curves were described well by power-law dependence:

$$A_P(C) = A_{P0}C^n \tag{3}$$

with power-law index $n = 0.49$ for the triglyceride and $n = 0.67$ for the oleic acid. The value of A_{P0} depends on the specific experimental conditions during chromatogram visualization (temperature, concentration of phosphomolybdic acid, etc.). Therefore, A_{P0} was determined in each experiment, by using an internal standard of known concentration. In this way, by using the known values of n and A_{P0}, we determined the concentrations of SFO and free fatty acids in the reaction mixture, at the end of the lipolysis reaction. Thus, we could evaluate the degree of triglyceride transformation.

2.9. Degree of Lipolysis. TG lipolysis could occur to DG and MG.[35] Therefore, one can define three different degrees of TG lipolysis: the overall degree of TG lipolysis, α; the degree of TG lipolysis to DG, β; and the degree of TG lipolysis to MG, γ. In this study, we consider the overall degree of TG lipolysis mostly, which is defined as

$$\alpha = \frac{C_{TG}^{INI} - C_{TG}^{END}}{C_{TG}^{INI}} \tag{4}$$

where the superscripts "INI" and "END" define the triglyceride concentrations in the beginning and at the end of the reaction, respectively.

2.10. Interfacial Tension Measurements. The interfacial tension of the surfactant solutions with sunflower oil (SFO), in the presence of all electrolytes in the reaction mixture (but without enzymes in the solution), was measured by the pendant drop method on a DSA 100 instrument (Krüss, Germany) at $T = 37$ °C, by using a thermostating chamber (Kruess, Germany). The obtained dependences for $\sigma(t)$ were converted to $\sigma(t^{-1/2})$ and fitted with linear dependence. From the intercept of the fit, the equilibrium value of the interfacial tension was determined and used for construction of the surface tension isotherms $\sigma(C_S)$, where C_S is the surfactant concentration in the aqueous phase.

3. EXPERIMENTAL RESULTS AND DISCUSSION

3.1. Triglyceride Transformation as a Function of Surfactant Concentration. This series of experiments was aimed at determining the dependence of TG transformation, α,

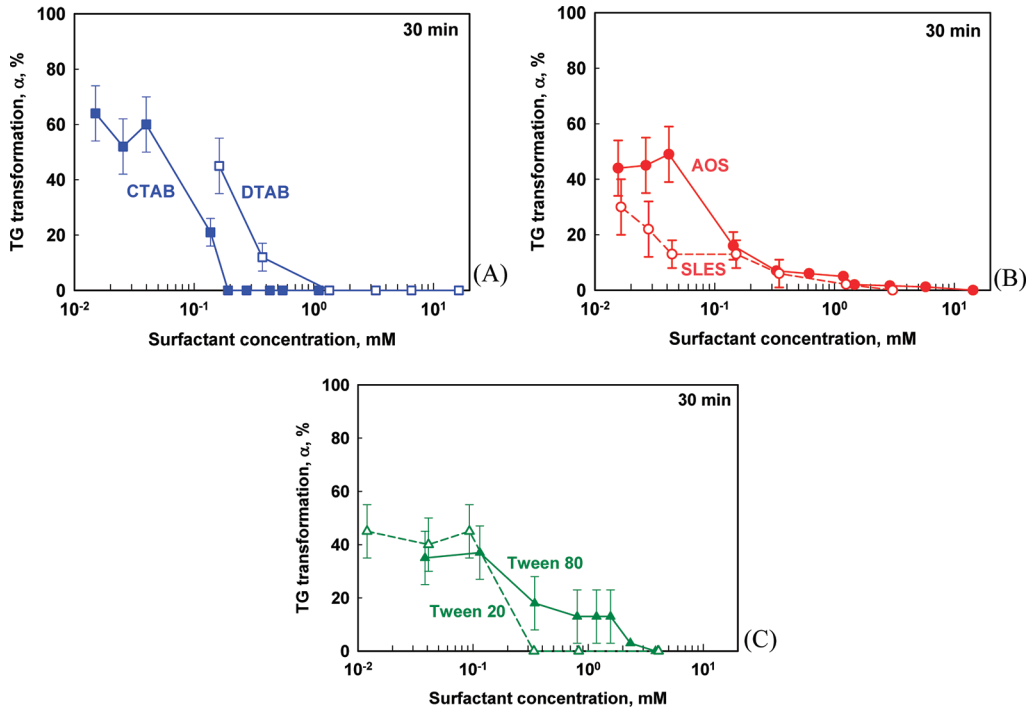

Figure 3. Triglyceride transformation, as a function of surfactant concentration, for surfactants with (A) cationic, (B) anionic, and (C) nonionic head groups. Experimental data are obtained after 30 min of reaction time.

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on the surfactant concentration in the reaction mixture, C_S, for series of six emulsifiers that differ in the charge of their head groups (nonionic, cationic, and anionic) and in the length of their hydrophobic tail (12−18 carbon atoms). All experiments were performed at fixed reaction time of 30 min. The initial emulsions for given surfactant had the same mean volume surface diameter: $d_{32} = 13 \pm 3$ μm for AOS, SLES, DTAB, and Tween 20 emulsions and 18 ± 5 μm for CTAB and Tween 80 emulsions. Thus, all studied emulsions had comparable mean drop size, so that the observed effects are related exclusively to the variations in the type and concentration of the surfactants studied. Note that similar values of d_{32}, at fixed oil concentration in the reaction mixture (0.2 wt % in all our experiments), correspond to similar drop surface area, available for oil lipolysis, which is the relevant quantity in the context of the lipolysis kinetics.

The obtained dependences $\alpha(C_S)$ for the cationic surfactants (CTAB and DTAB) are shown in Figure 3A. One sees that α remains approximately constant ($\approx$ 55%) for all solutions with $C_S \leq 0.04$ mM for CTAB emulsions. In the concentration range between 0.04 and 0.2 mM CTAB, α rapidly decreases from 55% down to zero and remains such at higher CTAB concentrations. Similarly, the degree of TG transformation for DTAB emulsions decreases from 45% down to zero when C_S increases from 0.2 to 1.4 mM. For all systems with negligible transformation, $\alpha \approx 0$, no visible spots were seen in the chromatograms for any of the possible reaction products (DG, MG, and FFA). Therefore, CTAB in concentration of 0.2 mM and DTAB in concentration of 1.4 mM are sufficient to suppress completely the TG transformation in the studied emulsions for at least 30 min of contact between the TG droplets and the enzyme−surfactant solution. Hereafter, we denote these concentrations as "threshold" surfactant concentration, C_{TR}, sufficient for enzyme inhibition in the first 30 min of TG−solution contact. One sees that C_{TR} for CTAB is around 7 times lower than that for DTAB.

The experimental results for the anionic surfactants (AOS and SLES) are shown in Figure 3B. Again, α decreases with increasing C_S and becomes zero above certain surfactant concentration C_{TR}. For these surfactants, however, the transition to suppressed TG transformation is more gradual; there is a wide range of concentrations, between 0.15 and 10 mM for AOS and between 0.15 and 2 mM for SLES, in which α gradually decreases from 20% to zero. Due to this wider range of surfactant concentrations corresponding to low α, the threshold surfactant concentrations for SLES and AOS are higher than those for CTAB and DTAB, despite the fact that the TG transformation is lower for the anionic surfactants (as compared to the cationic ones) in the range of low concentrations, $C_S < C_{TR}$ (cf. Figures 3A,B). Therefore, the anionic surfactants seem to be less efficient in blocking completely the enzyme activity but delay more efficiently the TG transformation at low surfactant concentrations. For further discussion of the differences between the various surfactants, see section 3.4.

The results obtained with the nonionic surfactants Tween 20 and Tween 80 are presented in Figure 3C. The threshold surfactant concentration above which the TG transformation becomes zero is ≈0.35 mM for Tween 20 and ≈4 mM for Tween 80. At $C_S \leq 0.1$ mM, the TG transformation for both emulsifiers is ≈40%. The degree of TG transformation decreases more steeply for Tween 20 and much more smoothly for Tween 80 emulsions. Therefore, the shape of the curve for $\alpha(C_S)$ for Tween 20 is similar to that for the cationic surfactants, whereas the dependence for Tween 80 is closer to that for the anionic surfactants.

It should be emphasized that for all systems in which $\alpha <$ 20%, no visible spot for MG was seen in the chromatograms, which means that the concentration of the formed MG in the first 20% of TG transformation was less than 1% (which is the minimal MG concentration that can be detected by the used analytical method). For the same systems, well visible spots for DG and FFA are seen in the chromatograms. Therefore, only the first stage in the transformation of the TG (to DG) is realized in the initial period of TG lipolysis under these conditions. In contrast, when the surfactant concentration was low and the degree of transformation was $\alpha >$ 20%, well visible spots for MG and DG were seen in the chromatograms.

The most important results from these experiments can be summarized as follows. (a) For all studied emulsifiers there are three well distinguished regions in the dependence $\alpha(C_S)$: at low C_S, $\alpha \approx$ const (region 1); in the intermediate concentration range, α decreases with the increase of C_S (region 2); and after certain threshold value of C_S, the TG transformation becomes zero (region 3). (b) The threshold value of C_S depends significantly on the type of used emulsifier. It is the smallest for CTAB- and Tween 20-stabilized emulsions ($C_{TR} \approx 0.3$ mM); intermediate for DTAB, Tween 80, and SLES ($C_{TR} \approx 1-3$ mM); and highest for AOS ($C_{TR} \approx 10$ mM). (c) For all studied emulsifiers, only DG and FFA are formed as reaction products when the TG transformation is $\alpha <$ 20%, which means that no MG is formed at low degree of TG transformation.

3.2. Kinetics of TG Transformation. All experimental results in the previous section were obtained after 30 min of reaction time. Therefore, some of them could be a result of kinetic effects, e.g., due to presence of induction period before lipase activation.[36−38] To check for such effects, we performed two types of experiments. In the first series of experiments we used the procedure described in section 2.4 (without keeping pH constant) and measured the TG transformation by TLC as a function of the reaction time up to 360 min. In the second series of experiments, we kept the pH constant by using the pH-stat instrument and measured by TLC the TG transformation after 120 min. All experiments in this part of the study were performed with the long-tail emulsifiers CTAB, Tween 80, and AOS, each of them taken at three different concentrations falling in regions 1, 2, and 3, respectively.

The experiments in region 3 were performed with 0.55 mM CTAB, 14 mM AOS, and 3.8 mM Tween 80. These surfactant concentrations were chosen to be well above the threshold concentrations, $C_S > C_{TR}$, above which the TG transformation becomes zero; see Figure 3. The obtained results with 0.55 mM CTAB and 3.8 mM Tween 80 showed no TG hydrolysis ($\alpha =$ 0) even after 6 h of reaction time; this result shows that the TG lipolysis is completely suppressed under these conditions, in the physiologically relevant time frame. The experimental results with AOS-stabilized emulsions showed formation of a small fraction of reaction products, but the TG transformation remained very low (less than 3%). Again, only FFA and DG were formed as reaction products in these emulsions, without detectable concentrations of MG.

Interestingly, for Tween 80 emulsions we observed formation of FFA, but there was not any detectable formation of MG and DG. The experiments with solutions of Tween 80 only (no triglycerides) showed that the enzyme hydrolyzes the molecules of Tween 80, which leads to formation of FFA as

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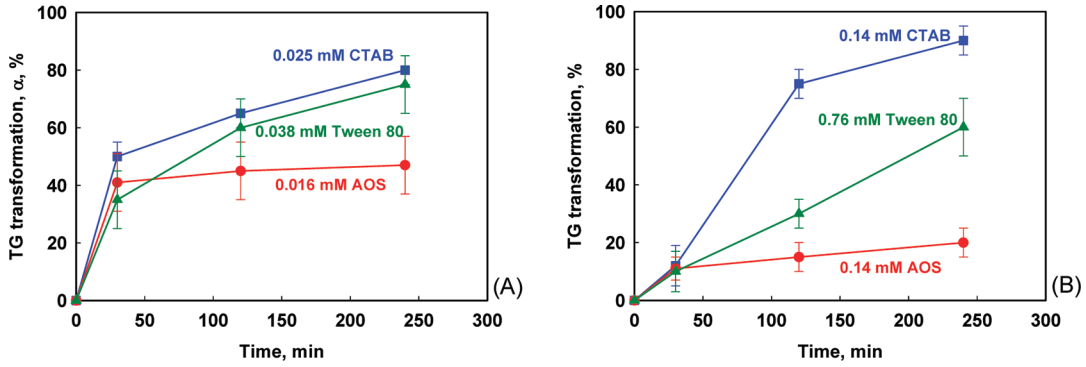

Figure 4. Triglyceride transformation, as a function of time, for emulsions stabilized by CTAB (squares), Tween 80 (triangles), and AOS (circles) at concentrations in (A) region 1 and (B) region 2. Experiments are performed with a starting pH of 7.5, which decreases slightly during the experiment, due to formation of free fatty acids.

reaction products. The concentration of the formed FFA in the experiments with and without TG emulsions showed practically the same kinetics of formation as FFA, which means that these acids were formed in the hydrolysis of the surfactant only. From the amount of formed FFA we could estimate that the enzyme hydrolyzes ≈0.3 mM of Tween 80 for 6 h, which is much less than the overall concentration of Tween 80 present in the studied emulsion (3.8 mM).

The kinetics of TG transformation in region 1 (low surfactant concentration) was studied with emulsions stabilized by 0.025 mM CTAB, 0.016 mM AOS, and 0.038 mM Tween 80 solutions. The degree of TG lipolysis was evaluated after reaction times of 30, 120, and 240 min. The obtained results are shown in Figure 4A. When the anionic surfactant AOS is present in the reaction mixture, the TG transformation increases steeply in the first 30 min (from 0 to 45%) and remains almost constant in the following 210 min. It should be mentioned that the concentration of the formed FFA increases with increasing the reaction time from 30 to 240 min. However, this increase in FFA concentration is related to the transformation of DG to MG (rather than to lipolysis of TG), because it is accompanied with a significant increase of the intensity of the MG spot at the expense of the DG spot.

The kinetics of the TG transformation for CTAB and Tween 80 emulsions was different from that observed with AOS; see Figure 4A. When CTAB and Tween 80 were used as emulsifiers, the initial steep increase of α in the first 30 min (from 0 to 40−50%) was followed by a slower but steady increase of α (up to 80%) during the next 210 min. For these emulsifiers, a simultaneous increase of the concentrations of DG and MG was observed during the entire reaction period, accompanying the increase of the FFA. From this series of experiments we conclude that the TG transformation in region 1 continues with the reaction time for CTAB and Tween 80 emulsions and reaches ≈75% after 240 min. Most probably, for these emulsions, a complete TG transformation will be achieved after a sufficiently long reaction period (ca. 8−10 h). In contrast, the kinetics of TG transformation in AOS emulsions is very slow after the first 30 min, and the main process is the transformation of the accumulated DG into MG.

A similar series of experiments was performed with surfactant concentrations in the intermediate region 2, with emulsions stabilized by 0.14 mM CTAB, 0.76 mM Tween 80, or 0.14 mM AOS. The obtained results are shown in Figure 4B. One sees that the TG transformation for AOS-stabilized emulsions remains rather low (α < 20%) in the entire period of the experiment (4 h). Even after 240 min, no visible spot for MG is seen, which means that the TG transforms to DG only under these conditions. The kinetics of TG transformation for CTAB and Tween 80 emulsions is different; see Figure 4B. For Tween 80 emulsions, α increases up to 60% after 240 min, whereas for CTAB emulsions the increase is even higher and a TG transformation of 90% is reached. Therefore, in the intermediate range of surfactant concentrations, the kinetics of TG transformation depends significantly on the type of surfactant used.

From the data presented in Figure 4, we cannot deduce the actual shape of the kinetic curves $\alpha(t)$, due to the limited number of experimental points available from the TLC analysis. For more precise characterization of the lipolysis kinetics, we used the pH-stat apparatus.[39,40] In this method, the pH is maintained constant by titrating the newly formed FFA by NaOH. An inherent problem of this method is that the solubility of the long-chain FFA, formed during the TG lipolysis, is relatively low at the physiologically relevant range of pH ≤ 7.5. Therefore, some of the formed FFA may remain dissolved in the oily phase or make a precipitate, which affects the interpretation of the experimental data.[41−44] In addition, even determining precisely the concentration of the FFA from the titrated NaOH, we cannot estimate directly the degree of TG transformation, because it is not known in advance what fraction of TG is transformed into MGs (with formation of two molecules of FFA) and the fraction transformed into DGs (where only one molecule of FFA is formed). Therefore, in the experiments with the pH-stat apparatus we determined the degree of release of FFA

$$\delta = \frac{C_{FFA}}{2C_{TG}^{INI}} \tag{5}$$

where C_{FFA} is the concentration of formed FFA and C_{TG}^{INI} is the initial TG concentration. The relation between α and δ depends on the ratio of the formed MG and DG. If the entire amount of TG is transformed into MG, $\alpha = \delta$. If the entire amount of hydrolyzed TG is transformed into DG, $\alpha = 2\delta$.

To account for the possible incomplete titration of the formed FFA in the pH-stat instrument, we considered the reaction

$$FFA + Na^+ = NaFA + H^+ \tag{6}$$

where FFA denotes the formed free fatty acids and NaFA are their sodium salts. These salts are also poorly soluble in the aqueous phase and may remain trapped in the oily phase (after

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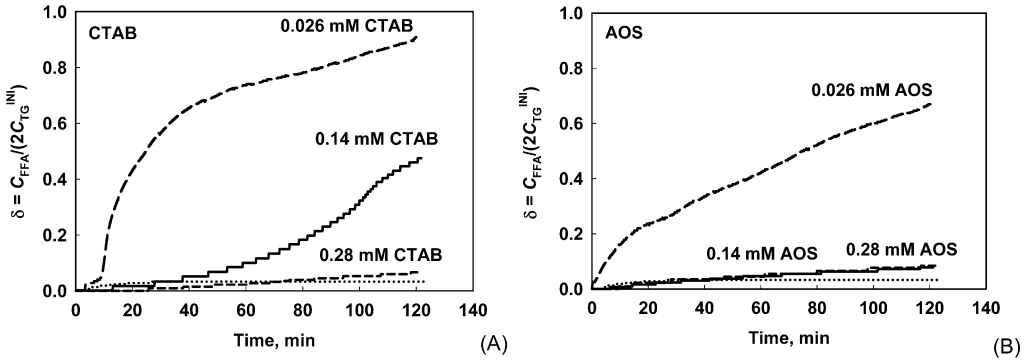

Figure 5. Degree of released free fatty acids, δ, as a function of time for emulsions stabilized by (A) CTAB and (B) AOS at concentrations in region 1 (long dash), region 2 (solid line), and region 3 (short dash). The dotted lines correspond to control experiments, performed in the absence of enzyme. All experiments are at fixed pH = 7.5 during the entire period of reaction.

being formed at the oil–water interface) or coprecipitate with the FFA to form acid–soap crystallites.[42,44] To describe quantitatively the titration of FFA in our systems, we determined the equilibrium constant of conversion of the FFA into their sodium salts by comparing the titrated FFA in the pH-stat experiment with the FFA in the same sample, as determined by TLC. From these experiments we determined the value $K \approx 10^{-6.78}$ for the equilibrium constant of reaction[6] (for the precise definition of K and its physical interpretation, see ref 41). By using this constant we recalculated the data from the pH-stat experiments and determined the kinetic curves $\delta(t)$ with very good time resolution; see Figure 5. We should mention that there is an additional difference between the data shown in Figures 3 and 4 (obtained by TLC) and the data shown in Figure 5 (obtained with pH-stat apparatus). The data shown in Figures 3 and 4 are obtained with a starting pH of 7.5 which gradually decreased in the course of the lipolysis reaction, whereas the data obtained with the pH-stat equipment are obtained at fixed pH = 7.5.

The obtained results for the CTAB emulsions are shown in Figure 5A. One sees that the shape of the curves $\delta(t)$ depends significantly on CTAB concentration in the reaction mixture. For the lowest studied concentration of 0.026 mM, a short induction time of ≈6 min is observed first, followed by a very steep increase of $\delta(t)$ in the next 30 min and a much slower increase in the following 90 min. The shape of the curve for 0.14 mM CTAB is different; δ remains very low in the first 40 min (long induction time), followed by a gradual increase in the period between 70 and 120 min. Therefore, we observed a clear induction period for the TG transformation at these two CTAB concentrations. The highest CTAB concentration studied in this series of experiment was 0.28 mM, where the degree of FFA conversion remained very low in the entire period of our experiments. TLC analysis of the final samples (after 120 min reaction time) showed no formation of spots for any of the reaction products (FFA, MG, or DG). The latter result indicates that the observed small consumption of NaOH in this experiment was due to other reactions, such as dissolution of CO_2 from the air into the reaction mixture. Indeed, control experiments performed in the absence of enzyme showed that we needed some small amount of NaOH to maintain pH = 7.5, even without lipolysis reaction; see the control points in Figure 5A.

The experimental results for AOS-stabilized emulsions are shown in Figure 5B. No induction period is seen for this surfactant and the values of δ are lower than those for CTAB emulsions in the entire experimental period. The dependences $\delta(t)$ at the higher concentrations of AOS showed a very low degree of transformation, and this result was confirmed by TLC analysis: $\alpha \approx 5\%$ after 120 min (cf. Figures 4 and 5).

Experiments with Tween 80 were also performed with this method, however, a significant amount of NaOH was consumed for titration of the fatty acids, formed from the enzymatic hydrolysis of the surfactant. As a result, the quantitative interpretation of the experimental results for the emulsions containing Tween 80 became very uncertain and, therefore, we do not show these results here.

Several conclusions can be drawn from these experiments. (1) The enzyme activity is almost completely blocked for all studied emulsifiers at $C_S > C_{TR}$ ($\alpha < 5\%$ even after 6 h). (2) TG transformation increases significantly with time for emulsions with $C_S < 0.1$ mM, and complete TG transformation is reached for CTAB- and Tween 80-stabilized emulsions after a certain period of time. (3) An induction period, in which the TG transformation is very low, is observed for CTAB-stabilized emulsions at 0.1 mM $< C_S < C_{TR}$.

The molecular mechanisms defining the various regimes of TG transformation are discussed in section 3.4, after presenting in the following section 3.3 results about the surface properties of the surfactants studied.

3.3. Interfacial Properties of the Emulsifiers. To characterize the interfacial properties of the emulsifiers used, we measured the interfacial tension of their solutions with SFO, σ, by using the pendant drop method. In these experiments we did not add enzyme, because the formed reaction products would change the interfacial tension during the experiments. The aqueous solutions contained all electrolytes present in the reaction mixtures, and the measurements were performed at 37 °C.

The interfacial properties of the studied surfactants were characterized with respect to (1) the equilibrium interfacial tension, σ_{EQ}, which is used to construct the interfacial tension isotherm, $\sigma_{EQ}(C_S)$, and to determine the critical micellization concentration of the surfactant, cmc, and (2) the adsorption constant, K_A, and maximal adsorption in the adsorption layer, Γ_∞, which were determined by interpreting $\sigma_{EQ}(C_S)$ isotherms by the Volmer equation (eq 7 below).

The measured values of $\sigma(t)$ at low concentrations of AOS and CTAB (below cmc) were represented as $\sigma(1/t^{1/2})$ for longer times and the equilibrium value σ_{EQ} was determined from the intercept $1/t^{1/2} \rightarrow 0$. This description of the experimental data implies that the adsorption is diffusion-

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limited at long adsorption times.[45,46] The obtained isotherms $\sigma_{EQ}(C_S)$ for AOS and CTAB are shown in Figure 6 and they

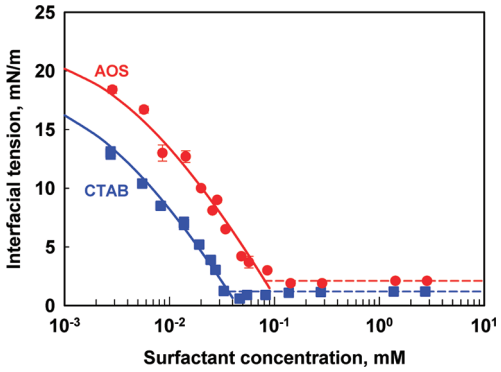

Figure 6. Equilibrium interfacial tension isotherms, measured by pendant drop method at 37 °C, for CTAB (squares) and AOS (circles). The lines below the cmc correspond to the best fits of the experimental data by the Volmer adsorption model (eq 7).

exhibit the regular sharp transition in the slope of the isotherms at the cmc. The respective values are cmc = 0.143 mM for AOS and cmc = 0.047 mM for CTAB. To extract information about the properties of the surfactant adsorption layers, we fitted the experimental data below the cmc with the Volmer equations:[45,46]

$$K_A C_S = \frac{\Gamma}{\Gamma_\infty - \Gamma} \exp\left(\frac{\Gamma}{\Gamma_\infty - \Gamma}\right) \quad (7a)$$

$$\sigma_{EQ} = \sigma_0 - kT\Gamma_\infty \left(\frac{\Gamma}{\Gamma_\infty - \Gamma}\right) \quad (7b)$$

A nonlinear fit was used with two adjustable parameters, the adsorption constant, K_A, and the maximum adsorption, Γ_∞ (σ_0 = 27 mN/m is the interfacial tension of the clean oil–water interface). The best fits are shown in Figure 6 as continuous curves, and the values of the obtained parameters are compared in Table 1, along with the calculated area per molecule in the

Table 1. Values of the Adsorption Constant (K_A), Maximum Adsorption (Γ_∞), and the Corresponding Area Per Molecule (A_∞), As Determined from the Volmer Adsorption Model (eq 7)

	K_A, × 10^6 M^{-1}	Γ_∞, × 10^{18} molecules/m^2	A_∞, nm^2
CTAB	0.39 ± 0.11	2.4	0.48
AOS	0.25 ± 0.10	2.1	0.47

dense adsorption monolayer, $A_\infty = 1/\Gamma_\infty$. The determined values A_∞ = 0.48 nm^2 for CTAB and A_∞ = 0.47 nm^2 for AOS are in a good agreement with those reported in the literature for similar molecules, TTAB[47] and SDS.[48]

Our attempt to interpret the experimental data for Tween 80 by the same approach was unsuccessful because the interfacial tension decreased with increasing C_S in the entire range of surfactant concentrations studied (see Figure IA in the Supporting Information). According to data reported in literature, the cmc of Tween 80 is ≈0.012 mM,[49] whereas we found in our measurements the interfacial tension to decrease continuously (from 8.4 to 3.8 mN/m) with the increase of C_S from 0.012 up to 10 mM. This means that the composition of the adsorption layer changes with the increase of surfactant concentration even above the cmc.

To check more carefully whether the observed decrease of $\sigma_{EQ}(C_S)$ for Tween 80 is affected by the extrapolation of the kinetic data used for determination of σ_{EQ} (based on the assumption for diffusion controlled adsorption), we determined the values of σ_{EQ} also by assuming that the kinetics of adsorption is barrier-controlled. In this case, $\sigma(t)$ is fitted by exponential equation.[45,46] The respective values of $\sigma_{EQ}(C_S)$ are presented in Figure IB in the Supporting Information. One sees similar trends for $\sigma_{EQ}(C_S)$, independently of the way used to determine σ_{EQ}; the interfacial tension decreases even above the cmc. The most probable explanation for this peculiar behavior of Tween 80 is that this is a technical product, containing a wide range of subcomponents with different numbers of ethoxy groups. With the variation of surfactant concentration, different components are preferentially adsorbing on the oil drop surface, thus changing the interfacial tension even above the cmc. The main conclusion here is that we cannot determine from these data a cmc value for Tween 80, because the adsorption layer changes its composition significantly in the entire range of surfactant concentrations studied. In the following discussion we use cmc ≈ 0.012 mM, as reported in the literature.[49]

The experimental interfacial tension isotherms of the other surfactants SLES, DTAB, and Tween 20 were used to determine the respective cmc values only. The results obtained with SLES solutions followed the typical curve for low molecular mass surfactants (like those shown in Figure 6) and cmc ≈ 0.05 mM was determined.

When the interfacial tension of the DTAB solutions with C_S > 0.9 mM was measured, we observed first a decrease of $\sigma(t)$, followed by a significant increase at longer adsorption times. Such kinetics of the interfacial tension (with minimum) has been reported in literature[50] for surfactants containing oil-soluble minor components (possibly remaining from the surfactant synthesis) that first adsorb on the interface from the aqueous solution and afterward dissolve in the oily phase (thus decreasing the adsorption and increasing the interfacial tension at long times). Another convincing indication for the presence of additional, strongly hydrophobic minor components in DTAB was the experimental fact that the isotherm $\sigma(C_S)$ passed through a deep minimum (≈4 mN/m) (see Figure II in the Supporting Information). Such a minimum has also been reported in the literature for surfactants containing hydrophobic impurities.[51] Therefore, the value of cmc ≈ 2 mM for DTAB was determined from the position of the minimum in the interfacial tension isotherm.

The experimental results for $\sigma_{EQ}(C_S)$ for Tween 20, as determined from the intercept of the kinetic curves plotted as $\sigma(1/t^{1/2})$, are presented in Figure III in the Supporting Information. For this surfactant we observed that the curves $\sigma_{EQ}(C_S)$ consist of two well-defined regions with different slopes. Note that the interfacial tension decreases with increasing C_S, even at the highest studied surfactant concentrations (see Figure III, Supporting Information). This means that the composition of the adsorption layer changes with the surfactant concentration in the entire range of concentrations studied (similarly to Tween 80). Such slow decrease in interfacial tension has been reported already in the literature for this surfactant.[52] From the intersection point of the two regions in the isotherms, we estimated cmc ≈ 0.08

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mM, a value that is in a reasonable agreement with the cmc reported in literature, ≈0.06 mM.[49]

From this series of experiments we can conclude that (1) AOS, CTAB, SLES, and Tween 20 have well-defined cmcs, above which the interfacial tension changes only slightly. The cmc values determined for these surfactants are 0.143 mM for AOS, 0.048 mM for CTAB, 0.05 mM for SLES, and 0.08 mM for Tween 20. (2) DTAB contains oil-soluble components, which leads to a well-pronounced minimum in the respective surface tension isotherm. The cmc is ≈2 mM for this surfactant. (3) Tween 80 contains a range of subcomponents with different numbers of ethoxy groups, which leads to a continuous decrease of the interfacial tension while increasing the surfactant concentration in the entire range of concentrations studied (up to 10 mM). cmc ≈ 0.012 mM is assumed for Tween 80 in the further discussion, as determined in the literature.[49]

3.4. Discussion and Further Experiments Aimed to Clarify Mechanism of Lipase Inhibition. In this section, we use the experimental results described above to discuss the various regimes of TG transformation and the physicochemical phenomena determining the value of C_{TR}. Also, several additional experiments are described, which were performed to check experimentally the proposed mechanistic explanations of the observed phenomena.

First we discuss the complete suppression of TG transformation, observed at high surfactant concentration ($C_S > C_{TR}$). There are two conceptually different mechanisms that can be proposed to explain this phenomenon (see Figure 7).

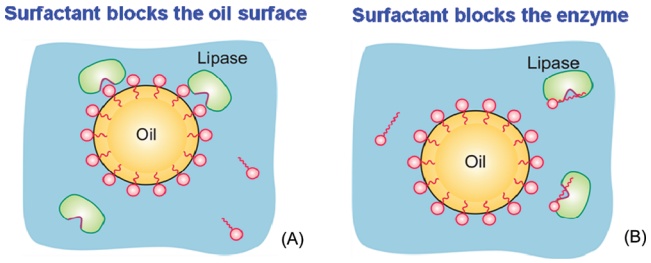

Figure 7. Schematic presentation of two possible mechanisms for enzyme inhibition by the used emulsifiers: (A) formation of dense adsorption layer on the drop surface, which prevents enzyme contact with the oil substrate and (B) surfactant adsorption around the active center of the enzyme molecules, which deactivates the enzyme.

The main difference between these two mechanisms is the location on which the surfactant molecules preferentially adsorb and block the lipolysis process: at the oil–water interface (thus preventing the lipase adsorption and contact with the TG molecules) or around the active center of the enzyme molecules (thus blocking this active center).

To clarify which of these mechanisms is operative in the systems studied here, we performed additional experiments with tributyrin as triglyceride substrate. Tributyrin has a sufficient molecular solubility in water to be hydrolyzed in dissolved molecular form. Therefore, by using this substrate we eliminate the oil–water interface, which is the location of the TG lipolysis in the typical experiments with SFO emulsions. Thus, we could check whether the enzyme center is still active at $C_S > C_{TR}$. These experiments were performed on a pH-stat apparatus and the titrated NaOH was used for assessment of the degree of tributyrin lipolysis.

As seen from the results shown in Figure 8, the enzyme is still rather active and hydrolyzes the soluble substrate tributyrin,

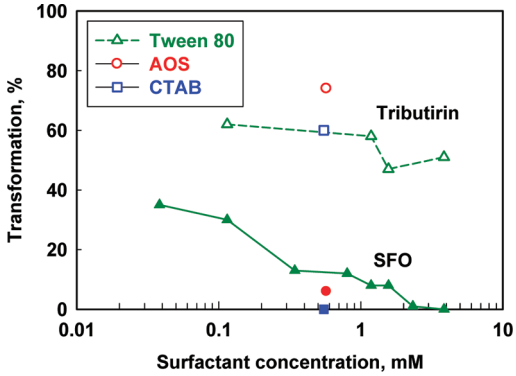

Figure 8. Degree of TG lipolysis of a SFO emulsion (full symbols) and of molecularly dissolved tributyrin (empty symbols), as a function of surfactant concentration for Tween 80 (triangles), CTAB (squares), and AOS (circles).

even at concentrations that are well above C_{TR} for all three emulsifiers tested, AOS, CTAB, and Tween 80. This result unambiguously demonstrates that the enzyme molecules are not denatured or blocked by the surfactant at $C_S > C_{TR}$. Thus, we have discarded the mechanism shown schematically in Figure 7B and supported the mechanism illustrated in Figure 7A.

The mechanism shown in Figure 7A implies that a relatively dense adsorption layer is formed on the drop surface, which prevents the direct contact of the enzyme molecules at $C_S > C_{TR}$. As far as this mechanism is related with the density of the surfactant adsorption layer, we checked how the lipase activity and TG transformation are affected by the surface coverage, $\theta = \Gamma/\Gamma_\infty$. From the values of K_A and Γ_∞ for CTAB and AOS, shown in Table 1, we calculated $\theta(C_S)$ for CTAB and AOS emulsions and converted the result into the dependence $\alpha(\theta)$, shown in Figure 9. One sees that the measured values of α after

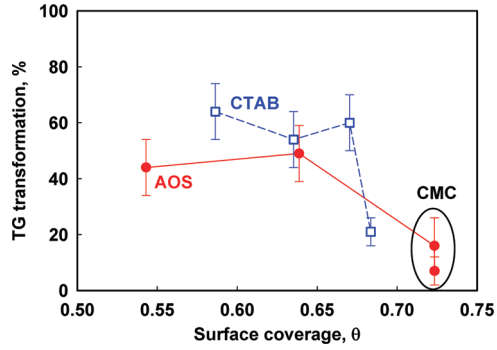

Figure 9. Degree of TG lipolysis, as a function of surface coverage, as calculated by the Volmer equation for CTAB (empty squares) and AOS (circles) stabilized emulsions.

30 min are ≈50% at $\theta < 0.7$, whereas a significant decrease of α is observed at $\theta \approx 0.7$. As shown in the previous section, the values $\alpha \approx 50\%$ at $\theta < 0.7$ (region 1, low surfactant concentrations) are due to the fact that the TG transformation is not completed yet for 30 min (see Figures 4 and 5). From all these results we can conclude that in region 1 (corresponding to $\theta < 0.7$) the enzyme can directly adsorb on the drop surface

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and the TG transformation starts immediately after placing the oil drops in contact with the enzyme solution (see Figure 5).

When denser adsorption layer with $\theta \approx 0.7$ is formed on the drops' surface (that is, around and above the cmc), the rate of TG hydrolysis is initially low, but the TG transformation may accelerate significantly after a certain induction period, as seen in Figure 5A. This regime of TG transformation corresponds to region 2 in our notation and its interpretation is as follows. The enzyme adsorption is relatively low in the beginning of the experiment, because an almost complete surfactant adsorption layer is formed. The adsorbed enzyme molecules, though of limited quantity, succeed to hydrolyze slowly TG molecules, thus leading to gradual accumulation of reaction products (FFA, MG, and DG). The formed FFA and MG are strongly surface active and get incorporated in the adsorption layer; this process is evidenced by the significant decrease of interfacial tension with the advance of TG transformation (data not shown). In the case of the cationic surfactant CTAB, the accumulation of the reaction products leads to acceleration of the lipolysis after the induction period (Figure 5A). For the anionic surfactant, a decrease in the rate of the TG lipolysis is observed at longer times in this regime (Figure 5B).

The specific values of the threshold surfactant concentration, C_{TR}, require additional discussion. According to the conventional description of the isotherms $\sigma_{EQ}(C_S)$, the surfactant adsorption and the value of θ should remain constant above the cmc).[45,46] Therefore, if the density of the adsorption layer on the drop surface was the only factor determining the degree of transformation $\alpha(C_S)$, we should expect that α would remain constant at $C_S >$ cmc, as far as θ does not change significantly above the cmc. In other words, the value of C_{TR} should be around the cmc of the respective surfactant. To check this reasonable assumption, we plot in Figure 10 the experimental

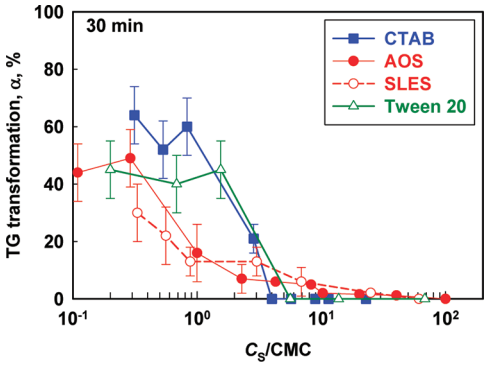

Figure 10. Degree of TG lipolysis as a function of C_S/cmc for emulsions stabilized by various surfactants.

results for AOS, CTAB, Tween 20, and SLES emulsions, as a function of C_S/cmc. One sees from this plot that complete blocking of the enzyme activity occurs only at surfactant concentrations that are much higher than the cmc; these are $C_{TR} \approx 5 \times$ cmc for the cationic and nonionic surfactants and $C_{TR} \approx 50 \times$ cmc for the anionic surfactants.

The latter experimental fact calls for more sophisticated mechanistic explanation of C_{TR}. The results from the interfacial tension measurements suggest that the dynamics of surfactant adsorption is another important factor (beside the equilibrium surface coverage θ) for achieving complete lipase inhibition. Let us explain first where the dynamic aspects emerge in our experiments. During the lipolysis, we stir the reaction mixture on a magnetic stirrer. Upon stirring, the emulsion drops are subject to hydrodynamic stresses, and therefore, the drops change their shape, which inevitably leads to variation of the drop surface area around an average value. Estimates, based on the analysis of the turbulent flow in the used containers,[53−55] and the related drop deformation[56] show that the drop surface area may vary ca. 1% around its average value, under the experimental conditions used in our in vitro model. During such fluctuations in the surface area, the enzyme competes with the surfactant molecules for adsorption on the newly created oil−water interface. When the surfactant concentration is high, the enzyme adsorption on the drop surface would be very improbable, because the surfactant molecules will be always faster in covering the formed vacancies in the adsorption layer (also, the surfactant has higher surface activity). In contrast, at low surfactant concentrations, there are some transient uncovered surface areas, where the enzyme molecules can adsorb and initiate the lipolysis reaction. Similar perturbations of the drop surface area are expected to occur in the GIT, where mechanical forces ensure the mixing and transport of the GIT juice.

Following these ideas, we compared the interfacial tensions of the surfactant solutions around cmc and at 5 × cmc, as a function of time. The obtained experimental results are plotted in Figure 11. One sees that the kinetics of surfactant adsorption is relatively slow for all surfactants studied, around their cmc. The characteristic time for surfactant adsorption is longer than ca. 12 s, which means that there is a long period allowed for adsorption of enzyme molecules on the free vacancies in the adsorption layer. However, when the surfactant concentration increases and reaches 5 × cmc for CTAB and 10 × cmc for the anionic surfactants, the kinetics of surfactant adsorption becomes very fast and we cannot measure the characteristic adsorption time by our methods, because it is shorter than 1 s. Therefore, the probability for enzyme adsorption at these higher surfactant concentrations is much lower, because the surfactant adsorption layer is permanently completed during the experiment.

The data for Tween 20, shown in Figure 11, require some additional comments. One sees that the interfacial tension of Tween 20 solutions changes slower, as compared to the other surfactants studied (see Figure 11). However, a careful look on the experimental data for Tween 20 shows a kink in the $\sigma(t)$ curve for $C_S \approx$ cmc (this kink is seen at $t \approx 100$ s), when the enzyme is still able to hydrolyze the substrate. In contrast, no such kink is observed at $C_S \geq C_{TR}$. Most probably, this kink in the curves reflects the formation of a dense adsorption layer on the drop surface, whereas the subsequent decrease of σ vs ln t is due to rearrangement of the (otherwise completed) adsorption layer. In other words, the observed slow decrease of σ at $C_S \geq C_{TR}$ is not caused by closing vacancies in the adsorption layer, which explains why the enzyme cannot adsorb on the drop surface and the lipolysis does not occur at such high surfactant concentrations.

The observed differences between the various types of surfactants (Figure 10) suggest that the charges of the surfactant head-groups are important. These charges may affect the interactions of the lipase and colipase with the surfactant adsorption layer.[4,16] Also, the different surfactant types may solubilize the reaction products (FFA and DG) in the surfactant micelles with different efficiency. For example, the anionic surfactants show a clear inhibitory effect below the cmc, followed by a much slower inhibition above the cmc, which

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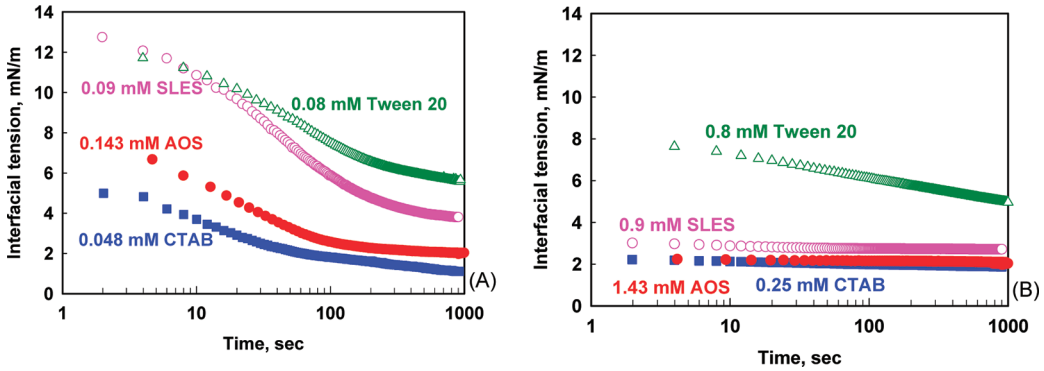

Figure 11. Kinetics of interfacial tension decrease for (A) concentrations around cmc and (B) concentrations around C_{TR}.

could involve the specific adsorption dynamics, as discussed above, or different solubilization efficiency of the surfactant micelles. The detailed analysis of these phenomena requires further experiments and more extended theoretical efforts.

4. CONCLUSIONS

Systematic experimental study of the effects of (1) type of used emulsifier, (2) emulsifier concentration, and (3) reaction time, on the degree of fat lipolysis, α, is performed with sunflower oil-in-water emulsions. Three different types of emulsifier, anionic (SLES and AOS), nonionic (Tween 20 and Tween 80), and cationic (DTAB and CTAB), are studied. The main conclusions can be summarized as follows.

For all systems studied, three well-defined regions are observed in the dependence degree of fat lipolysis vs surfactant concentration, $\alpha(C_S)$, when the latter is scaled with the critical micelle concentration (Figures 3, 4, 10):

- Region 1: At $C_S \leq$ cmc, for all emulsifier studied α reaches 0.4−0.6 after 30 min and increases up to 0.8−0.9 after 240 min.
- Region 2: At $C_S \approx 3 \times$ cmc, $\alpha \approx 0.1$−0.2 after 30 min for all used emulsifiers and increases steeply up to 0.8−0.9 after 120 min for the cationic and nonionic surfactants, whereas it remains low for the anionic surfactants ($\alpha \approx 0.2$).
- Region 3: At C_S above certain threshold value, C_{TR}, $\alpha \approx 0$ for all emulsifiers, for reaction time up to 480 min. $C_{TR} \approx 5 \times$ cmc for the nonionic and cationic surfactants and $C_{TR} \approx 50 \times$ cmc for the anionic surfactants (Figure 10).

Experiments with the water-soluble substrate tributirin showed that the enzyme is able to hydrolyze this substrate, even at surfactant concentrations well above C_{TR} (Figure 8). In this way we demonstrated that the enzyme molecules are not denatured or blocked by the surfactant above its C_{TR}. Thus, we proved that the major mechanism of enzyme inhibition is the formation of a dense surfactant adsorption layer on the oil drop surface, which displaces the enzyme from a direct contact with the interface water-insoluble triglycerides (Figure 7A).

By characterizing the interfacial activity of the studied surfactants we revealed that, in region 1, the emulsifier molecules are unable to prevent the adsorption of enzyme molecules on the drop surface. In region 2, an almost complete adsorption layer is formed on the drop surface ($\theta \approx 0.7$), but due to the slower kinetics of emulsifier adsorption, there is some probability for enzyme adsorption on the drop surface during emulsion agitation. This eventually leads to a high degree of lipolysis, up to 0.8, in the longer time scale (above 120 min). In region 3, the rapidly adsorbing surfactant molecules block completely the enzyme contact with the TG surface and no lipolysis is observed.

In the second paper of this series[27] we study systematically the interactions between the same emulsifiers and the bile salts, to clarify the mechanism of lipase−colipase reactivation by the bile salts.

■ ASSOCIATED CONTENT

Supporting Information

Figure with equilibrium interfacial tension at the SFO−water interface, as a function of Tween 80 concentration. (The interfacial tension is determined under the assumptions of (A) diffusion control and (B) barrier control of adsorption.) Experimental results for interfacial tension at the SFO−water interface, as a function of DTAB concentration and as a function of Tween 20 concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AOS, α-olefin sulfonate
cmc, critical micelle concentration
CTAB, cetyltrimethylammonium bromide
DG, diglycerides
DGDG, digalactosyldiacylglycerol
DPPC, dipalmitoylphosphatidylcholine
DTAB, dodecyltrimethylammonium bromide
GIT, gastrointestinal tract

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MG, monoglycerides
OA, oleic acid
PL, pancreatic lipase
SFO, sunflower oil
SLES, sodium lauryl ether sulfate
TG, triglycerides

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