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New Mathematical Model for Interpreting pH-Stat Digestion Profiles: Impact of Lipid Droplet Characteristics on in Vitro Digestibility

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The pH-stat method is commonly used to characterize the in vitro digestibility of lipids under simulated small intestine conditions. This method measures the fraction of free fatty acids (FFA) released from triacylglycerols over time. A new mathematical model has been developed to characterize the FFA versus time profiles generated by the pH-stat method, which can be used to quantify the influence of physicochemical parameters on the rate (k) and extent (ϕ_{max}) of lipid digestion. In this model, k is the amount of FFA produced per unit time per unit surface area, whereas ϕ_{max} is the maximum fraction of digestible FFAs released. This model is used to quantify the influence of lipid droplet characteristics (size, concentration, composition, and emulsifier type) on the digestion of emulsified lipids. The rate (k) of lipid digestion increased with decreasing lipid content (from 2.5 to 0.5 wt %), increasing droplet diameter (from $d = 200-15000$ nm), and decreasing fatty acid molecular weight (MCT versus corn oil). The extent (ϕ_{max}) of lipid digestion was also considerably less for corn oil than for MCT. The rate and extent of lipid digestion did not depend strongly on initial emulsifier type: β-lactoglobulin, Tween 20, lecithin, or lyso-lecithin. These results are interpreted in terms of differences in the concentrations of reactants, products, catalysts and cofactors at the lipid droplet surfaces during digestion, for example, triacylglycerols, emulsifiers, FFA, lipase, and bile salts. This model provides a useful means of quantifying the influence of specific parameters on lipid digestion using the pH-stat method.

KEYWORDS: Emulsions; lipid digestion; lipase; calcium; calcium binding; pH-stat

INTRODUCTION

There is growing interest in understanding and controlling the digestibility of emulsified lipids as they pass through the human gastrointestinal (GI) tract (*1−5*). This knowledge is being used by the pharmaceutical industry to rationally design emulsion-based delivery systems to carry biologically active agents to specific locations within the GI tract and release them at a controlled rate (*6, 7*). Analogous systems are also being developed by the food industry to encapsulate, protect, and deliver bioactive food components (nutraceuticals) (*4, 5, 8−11*). These delivery systems have been proposed as a means of increasing the bioavailability of highly lipophilic nutraceuticals or as a means of delivering functional food ingredients to specific locations within the GI tract, such as the colon for chemopreventative agents. Emulsion-based delivery systems with controlled stability and digestibility within the GI tract have also been proposed to be an effective method of controlling appetite and therefore combating obesity (*12−16*). In this case, studies have found that emulsions which remain stable to gravitational separation in the stomach and/or which have a delayed digestion in the small intestine can stimulate the release of gut hormones that induce satiety and reduce food intake. Consequently, there is great interest in the development of analytical tools that can be used to establish the major physicochemical and structural factors that affect lipid digestion and absorption under conditions that simulate the human GI tract. These tools range from physicochemical measurements using in vitro digestion tests, to cell culture models, to animal feeding studies, and ultimately to human trials (*4, 17, 18*).

The pH-stat method is an analytical tool that is finding increasing use within pharmaceutical and food research for the in vitro characterization of lipid digestion under simulated small intestinal conditions (*19−22*). The pH-stat method is based on measurements of the amount of free fatty acids (FFAs) released from lipids, usually triacylglcyerols (TAGs), after lipase addition at pH values close to neutral. The lipid-containing sample to be analyzed is placed within a temperature-controlled reaction chamber that contains simulated small intestinal fluid (SSIF). The SSIF typically includes appropriate concentrations of the major digestive components known to influence lipid digestion, such as lipase, colipase, bile salts, phospholipids, and mineral ions. The lipase in the SSIF catalyzes lipid digestion leading to the generation of two FFAs and one monoacylglycerol (MAG) per TAG molecule. The concentration of alkali (NaOH) that must then be titrated into the digestion cell to neutralize any FFA produced by lipid digestion, and thereby maintain the pH at the

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initial preset value (e.g., pH 7.0), is recorded versus time. In principle, the pH-stat method is relatively simple and rapid to carry out and enables comparison of different lipid formulations under similar experimental conditions. This technique can therefore be used to rapidly screen the impact of different physicochemical factors expected to affect lipid digestion.

In the present study, we develop a relatively simple mathematical model that can be used to quantify the FFAs released versus time profiles obtained from pH-stat instruments. By fitting this model to the experimental data it is possible to extract a parameter that is related to the rate of digestion (k) and another that is related to the extent of digestion (ϕ_{max}). We also intend to demonstrate the usefulness of this model by using it to characterize the impact of various droplet characteristics on the rate and extent of lipid digestion: droplet size, droplet concentration, emulsifier type, and droplet composition.

MATERIALS AND METHODS

Materials. Powdered β-lactoglobulin (BLG) was obtained from Davisco Foods International (lot JE 002-8-415, Le Sueur, MN). Tween 20 (T20) was purchased from MP Biomedicals LLC (lot 1131K). Lecithin (Lec) was obtained from American Lecithin Co. (lot R550000798). Deoiled enzyme-modified soy lecithin (Lyso Lec) was obtained from The Solae Co. (lot R550000854). Medium-chain triglyceride (MIGLYOL 812N) (MCT) was purchased from Sasol Germany GmbH (Witten, Germany). Corn oil was purchased from a local supermarket and used without further purification. Lipase from porcine pancreas, type II (L3126), and bile extract (porcine, B8613) were purchased from Sigma-Aldrich (St. Louis, MO). Calcium chloride ($CaCl_2 \cdot 2H_2O$) and sodium chloride (NaCl) were obtained from Fisher Scientific. Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Sigma. Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, Dubuque, IA) was used for the preparation of all solutions.

Solution Preparation. Emulsifier solutions were prepared by dispersing 1.0 wt % of emulsifier (BLG, T20, Lec, Lyso Lec) into 5 mM phosphate buffer solution and stirring for at least 2 h. The solutions were kept overnight at 4 °C to ensure complete hydration. NaCl and $CaCl_2$ solutions were prepared by dissolving a certain amount of powder into phosphate buffer and double-distilled water, respectively.

Emulsion Preparation. The basic procedure for emulsion preparation consisted of homogenizing 10 wt % oil phase with 90 wt % aqueous phase (1.0 wt % emulsifier, pH 7.0) using a high-speed blender for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Basle, Switzerland) followed by passage through a high-pressure homogenizer (Microfluidics M-110Y, F20Y 75 μm interaction chamber, Newton, MA) at 9000 psi for five passes. For certain experiments, some of these parameters were varied:

Droplet Composition. BLG-stabilized emulsions with different droplet compositions were prepared by using different oil phases, that is, either corn oil or medium-chain triglycerides.

Droplet Size. BLG-stabilized emulsions with different mean droplet sizes were prepared by varying the homogenization conditions: (i) only the high-speed blender was used for 2 min (coarse emulsion, CE); (ii) a high-speed blender and a microfluidizer at 5000 psi for two passes were used (medium emulsion, ME); (iii) a high-speed blender and a microfluidizer at 9000 for five passes were used (fine emulsion, FE); (iv) a blender and a microfluidizer at 14000 for 10 passes were used (ultrafine emulsion, UFE).

Emulsifier Type. Emulsions containing droplets coated with different types of emulsifier were prepared by using different emulsifier solutions: BLG, T20, Lec, or Lyso Lec.

The final compositions of the components in the digestion cell are listed in **Table 1**.

Microstructure Changes. Changes in emulsion microstructure during digestion were characterized to assess whether the assumptions underlying the mathematical model were appropriate. The change in microstructure was determined by comparing the properties of (i) *initial emulsion*, the emulsion before adding SSIF (pH 7.0); *predigested emulsion*, initial emulsion after addition of SSIF without lipase; and *digested emulsion*, the emulsion after digestion in SSIF containing lipase. Emulsions were prepared and then kept overnight at ambient temperature before analysis.

Table 1. Parameters Describing the Rate and Extent of Digestion in Oil-in-Water Emulsions Measured by pH-Stat Method

	d_0 (nm)	k ($\mu mol\ s^{-1}\ m^{-2}$)	ϕ_0 (%)	$t_{1/2}$ (min)
Effect of Droplet Composition (BLG Stabilized)				
MCT	300	0.93	96.9	2.80
corn oil	280	0.46	45.6	2.84
Effect of Droplet Concentration (BLG Stabilized, MCT)				
0.5 wt %	300	5.12	101.0	0.51
1.0 wt %	300	2.66	103.6	0.98
2.5 wt %	300	0.82	100.3	3.20
Effect of Droplet Size (BLG Stabilized, MCT)				
UFE	195	0.65	93.5	2.62
FE	300	0.93	97.1	2.79
ME	395	1.40	91.8	2.46
CE	14,700	3.93	116.3	32.5
Effect of Emulsifier Type (MCT)				
BLG	300	0.92	97.1	2.85
Tween 20	240	0.82	92.9	2.60
lecithin	260	0.81	92.9	3.00
lyso-lecithin	280	0.70	92.1	3.22

The concentrations of lipid, bile, $CaCl_2$, and NaCl in the predigested emulsion were the same as those in the pH-stat digestion vessel.

In Vitro Digestion Model. The in vitro digestion model (pH stat) used in this study was a modification of those described previously (23, 24). The basic procedure used was (i) 19.0 mL of emulsion containing a certain amount of oil was placed into a glass beaker that was placed in a water bath at 37.0 °C for 10 min and then adjusted to pH 7.0 using NaOH or HCl solution; (ii) 15.0 mL of bile extract solution (750 mg of bile extract dissolved in phosphate buffer, pH 7.0, 37.0 °C), 1.0 mL of $CaCl_2$ solution (375 mM $CaCl_2$ in double-distilled water, 37.0 °C), and 1.0 mL of NaCl solution (5625 mM NaCl in double-distilled water, 37.0 °C) were added to the emulsion under stirring, and the system was adjusted back to pH 7.0 if required; (iii) 1.5 mL of freshly prepared lipase suspension (90 mg of lipase powder dispersed in phosphate buffer, pH 7, 37.0 °C) was added to the above mixture. The final composition of the sample in the digestion cell was therefore 0.5% lipid, 20 mg/mL bile extract, 2.4 mg/mL lipase, 10 mM $CaCl_2$, and 150 mM NaCl unless otherwise stated. A pH-stat automatic titration unit (Metrohm, USA Inc.) was then used to automatically monitor the pH and maintain it at pH 7.0 by titrating an appropriate concentration of NaOH solution into the digestion cell. Unless otherwise stated, the titration solution was 0.15 M NaOH solution. The volume of NaOH added to the emulsion was recorded and used to calculate the concentration of free fatty acids generated by lipolysis.

Particle Size and ζ-Potential Measurements. Particle size and ζ-potential were determined using a commercial dynamic light scattering and microelectrophoresis device (Nano-ZS, Malvern Instruments, Worcestershire, U.K.). The samples were diluted 100 times in appropriate buffer solution at room temperature before measurement. The particle size data are reported as the Z-average mean diameter, whereas the particle charge data are reported as the ζ-potential.

The particle size distribution of the emulsions was also measured using a laser light scattering instrument (Mastersizer, Malvern Instruments). This instrument measures the angular dependence of the intensity of laser light (λ = 632.8 nm) scattered by a dilute emulsion and then finds the particle size distribution that gives the best agreement between theoretical predictions (Mie theory) and experimental measurements. To avoid multiple scattering effects, emulsions were diluted to a droplet concentration of approximately 0.005 wt % using buffer solution at the pH of the sample. A refractive index ratio of 1.08 was used in the calculations of the particle size distribution. The particle size measurements are reported as the surface-weighted mean diameter $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of droplets of diameter d_i.

Optical Microscopy. The microstructures of selected emulsions were observed using an optical microscope (Nikon Eclipse E400, Nikon Corp.,

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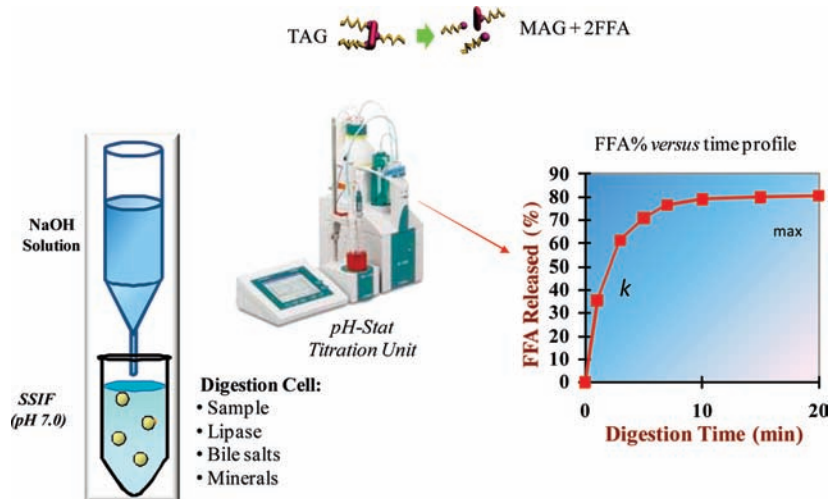*J. Agric. Food Chem.,* Vol. 58, No. 13, 2010 **8087**

Figure 1. Schematic representation of the pH-stat in vitro digestion model. The concentration of alkali required to maintain the pH at a constant preset level is measured and used to calculate the concentration of FFA released.

Tokyo, Japan). Emulsion samples were thoroughly mixed in a glass test tube before analysis. A drop of emulsion was then placed on a microscope slide, covered by a coverslip. An image of the sample was acquired using digital image processing software (Micro Video Instruments Inc., Avon, MA) and stored on a personal computer.

A Nikon confocal microscope (C1 Digital Eclipse) with a 60× oil immersion objective lens was used to capture the confocal images. Nile red (a fat-soluble fluorescent dye) was excited with 488 nm argon laser line. The fluorescence emitted from the sample was monitored using a fluorescence detector (515/30) with a pinhole size of 150 μm. The resulting images consisted of 512 × 512 pixels, with a pixel size of 414 nm and a pixel dwell time of 61.44 μs.

Data Analysis. All experiments were performed at least twice on freshly prepared samples. The results were then reported as averages and standard deviations of these measurements.

MATHEMATICAL MODELING OF DIGESTION PROFILES

A schematic diagram highlighting the principles underlying the pH-stat method is shown in **Figure 1**. The sample to be analyzed is placed in a digestion cell containing SSIF. FFA are released from the sample when lipase in the SSIF interacts with triacylglycerols in the digestion cell. Each triacylglycerol molecule generates two FFAs when fully digested; hence, the fraction of FFA released from a sample can be calculated from knowledge of the total amount of triacylglycerols originally present within the digestion cell and the amount of FFAs released after a particular digestion time. The percentage of FFAs released is calculated from the number of moles of alkali (NaOH) required to neutralize the FFA divided by the number of moles of FFA that could be produced from the triacylglycerols if they were all digested (assuming two FFA produced per triacylglycerol molecule):

$$\%FFA = 100 \times \left(\frac{V_{NaOH} \times m_{NaOH} \times M_{lipid}}{w_{lipid} \times 2} \right) \quad (1)$$

Here V_{NaOH} is the volume of sodium hydroxide required to neutralize the FFA produced (L), m_{NaOH} is the molarity of the sodium hydroxide solution used (in M), w_{lipid} is the total mass of triacylglycerol oil initially present in the digestion cell (in g), and M_{lipid} is the molecular mass of the triacylglycerol oil (in g mol^{-1}). Blanks were carried out in the absence of oil and subtracted from the reported values. The data generated by the pH-stat method consist of a curve of %FFA released versus time.

If it is assumed that the lipid droplets are contained within a digestion cell of volume V_E and that the rate of FFA release is proportional to the surface area per unit volume of the oil droplets, then

$$\frac{dm_d}{dt} = -kS_d V_E \quad (2)$$

Here dm_d/dt is the number of moles of FFA leaving the droplets per unit time, k is a rate constant equal to the number of moles of FFA leaving the droplets per unit time per unit surface area (mol s^{-1} m^{-2}), and S_d is the droplet surface area per unit emulsion volume. We assume that the total number of droplets in the system remains constant but that their diameter decreases as they are digested and some of the lipids leave. The specific surface area of the droplets is then given by

$$S_d(t) = n_0 \pi \, d(t)^2 \quad (3)$$

where n_0 is the number of droplets per unit volume of emulsion and $d(t)$ is the droplet diameter at time t. The total number of droplets per unit volume is given by

$$n_0 = \frac{6\phi_0}{\pi d_0^3} \quad (4)$$

where ϕ_0 is the initial droplet volume fraction and d_0 is the initial droplet diameter. The droplet diameter at a particular time can be related to the fraction of FFA released from the droplets due to digestion. The ratio of the droplet volume during digestion to the initial droplet volume is given by

$$\frac{V_d(t)}{V_0} = \frac{\frac{1}{6}\pi d(t)^3}{\frac{1}{6}\pi d_0^3} = \frac{m_d}{m_0} \quad (5)$$

where m_d is the number of moles of digestible FFA remaining within the droplets at time t and m_0 is the total amount of digestible FFA present within the droplets before digestion. Hence

$$d(t) = d_0 \sqrt[3]{\frac{m_d}{m_0}} \quad (6)$$

Substituting eqs 3−6 into eq 2 leads to

$$\frac{dm_d}{dt} = -k \left(\frac{6\phi_0}{d_0} \right) \left(\frac{m_d}{m_0} \right)^{3/2} V_E \quad (7)$$

This equation can be integrated to give the following expression, by taking into account that $m_d = m_0$ at $t = 0$:

$$\frac{m_d}{m_0} = \left(1 + \frac{3k\phi_0 t V_E}{d_0 m_0}\right)^{-2} \tag{8}$$

Let Φ be the ratio of the digestible FFA released to the total amount of digestible FFA originally present:

$$\Phi = 1 - \frac{m_d}{m_0} = 1 - \left(1 + \frac{3k\phi_0 t V_E}{d_0 m_0}\right)^{-2} \tag{9}$$

The initial volume fraction of oil can be related to the number of moles of FFA originally present in the droplets

$$\phi_0 = \frac{w_d}{V_E \rho} = \frac{m_0 M}{2 V_E \rho} \tag{10}$$

where w_d is the initial mass of the oil droplets, M is the molecular weight of the triglyceride oil, ρ is the density of the oil, and the factor 2 takes into account that two FFA are produced per triglyceride molecule. This expression can be substituted into eq 8 to give

$$\Phi = 1 - \left(1 + \frac{3kMt}{2d_0\rho_0}\right)^{-2} \tag{11}$$

It should be noted that this equation does not depend on the initial lipid concentration, but it does depend on the initial droplet size. Finally, this equation assumes that all of the FFA will be released from the droplets. In many cases, only a fraction (ϕ_{max}) of the FFA is released because the lipolysis reaction is inhibited. The equation can therefore be modified to take this into account:

$$\Phi = \phi_{max}\left(1 - \left(1 + \frac{3kMt}{2d_0\rho_0}\right)^{-2}\right) \tag{12}$$

The FFA versus digestion time profile resulting from a lipolysis reaction can then be characterized in terms of just two parameters, ϕ_{max} and k, by fitting the above equation to the experimental data and finding the values that minimize the difference between the data and the model. The ϕ_{max} parameter provides a measure of the extent of the digestion reaction, whereas the k parameter provides a measure of the rate of the digestion reaction. Another parameter that is also useful is the "digestion half-time" ($t_{1/2}$), which can be defined as the time required to reach the halfway point of lipid digestion. In this case, $\Phi = 0.5\phi_{max}$, so that eq 12 can be rearranged to give

$$t_{1/2} = \frac{2d_0\rho_0}{3kM} \times (\sqrt{2} - 1) \tag{13}$$

In our study, we used MathCad to find the values of ϕ_{max} and k that gave the best fit between the experimentally determined values of %FFA released versus time profiles and eq 12. For corn oil we used $\rho_0 = 910$ kg m^{-3} and $M = 0.800$ kg mol^{-1}, whereas for MCT we used $\rho_0 = 945$ kg m^{-3} and $M = 0.500$ g mol^{-1}.

RESULTS AND DISCUSSION

The overall objective of these experiments was to establish the utility of the new mathematical model for characterizing the FFA release profiles generated by the pH-stat digestion method. In addition, we wanted to use the model to quantify the influence of various lipid droplet characteristics on the rate and extent of digestion.
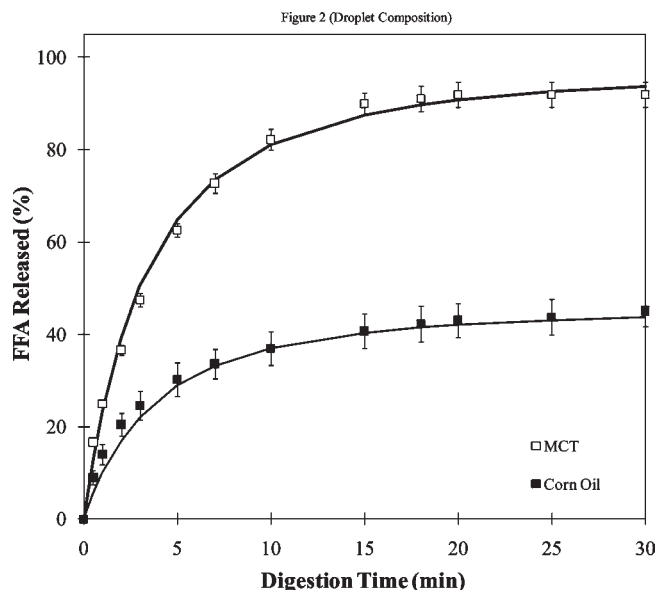

Figure 2. Influence of lipid droplet composition (corn oil or medium-chain triglycerides) on the pH-stat digestion profiles of 2.5 wt % oil-in-water emulsions stabilized by β-lactoglobulin. The curves are the best fits to the experimental data predicted using the mathematical model. Parameters derived from these curves are included in **Table 1**.

Effect of Droplet Composition. Initially, we examined the impact of lipid type on the rate and extent of digestion because different kinds of lipids are commonly used to prepare delivery systems in both the food and pharmaceutical industries. Oil-in-water emulsions stabilized by β-Lg were prepared that had similar droplet sizes ($d_{32} \sim 290$ nm) and droplet concentrations (2.5 wt %) using either corn oil or medium-chain triglyceride (MCT) as the lipid phase. MCT was used as an example of a triacylglycerol oil containing mainly medium-chain fatty acids, whereas corn oil was used as an example with mainly long-chain fatty acids. These emulsions were then tested with the pH-stat digestion model to obtain their FFA released versus time profiles (**Figure 2**).

The rate and extent of lipid digestion in the emulsions were clearly higher for MCT than for corn oil (**Figure 2**). These results are in agreement with earlier studies that have demonstrated that lipids containing medium-chain fatty acids are digested more rapidly than those containing long-chain fatty acids (3, 25). The mathematical model developed under Materials and Methods was used to fit the experimental %FFA versus time profiles to estimate the rate (k), extent (ϕ_{max}), and half-time ($t_{1/2}$) of lipid digestion (**Table 1**). There was a good fit between the mathematical model and the experimental data for both systems (**Figure 2**), suggesting that the model was useful for describing pH-stat digestion profiles. The parameters predicted using the model indicated that the rate of lipid digestion was appreciably faster for MCT ($k = 0.93$ μmol s^{-1} m^{-2}) than for corn oil ($k = 0.46$ μmol s^{-1} m^{-2}), whereas the extent of lipid digestion was much higher for MCT ($\phi_{max} = 97\%$) than for corn oil ($\phi_{max} = 46\%$). These k values are equivalent to about 0.6 and 0.3 FFA molecules being generated s^{-2} nm^{-2} for MCT and corn oil, respectively. For reference, the area occupied by a free fatty acid molecule at an oil–water interface is around 1 nm^2 (26). Previously, it has been reported that lipid digestion may be inhibited when 110–120 μmol m^{-2} of FFA accumulate at an oil–water interface (1). If we assumed that all of the FFA remained at the droplet surface in the corn oil emulsions, then it would take approximately 4–5 min to reach this level, that is, 110–120 μmol m^{-2}/0.46 μmol s^{-1} m^{-2}.

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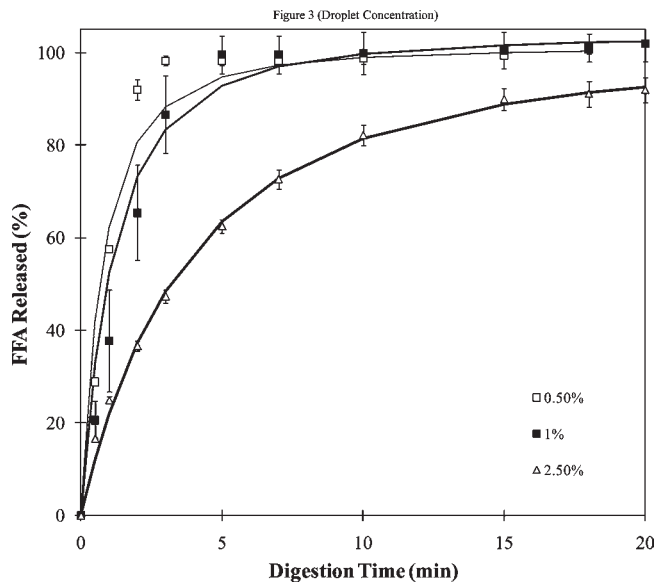*J. Agric. Food Chem.,* Vol. 58, No. 13, 2010 **8089**

Figure 3. Influence of initial lipid concentration on the pH-stat digestion profiles of MCT oil-in-water emulsions stabilized by β-lactoglobulin. The curves are the best fits to the experimental data predicted using the mathematical model. Parameters derived from these curves are included in **Table 1**.

Figure 4. Influence of initial lipid droplet diameter on the pH-stat digestion profiles of MCT oil-in-water emulsions stabilized by β-lactoglobulin. The curves are the best fits to the experimental data predicted using the mathematical model. Parameters derived from these curves are included in **Table 1**.

This value is fairly similar to the time when the FFA released reached a constant level in the corn oil emulsions (**Figure 2**).

This faster rate and higher extent of lipid digestion in the MCT emulsion can be attributed to the fact that medium-chain FFAs have a higher water dispersibility than long-chain FFAs (*3, 25*). The medium-chain FFAs produced during digestion of MCT are able to migrate rapidly into the surrounding aqueous phase and therefore do not inhibit the interfacial lipase reaction. On the other hand, the long-chain FFAs produced by corn oil tend to accumulate at the oil−water interface and inhibit lipase activity until they are removed by being solubilized in micelles or precipitated by calcium ions.

Effect of Lipid Droplet Concentration. The amount of emulsified fat present within the small intestine depends on the total quantity of food ingested, the fat content of that food, and the physicochemical processes occurring in the mouth, stomach, and small intestine (*4, 5, 27*). The influence of fat droplet concentration on the rate and extent of lipid digestion was therefore investigated. Different amounts of MCT oil-in-water emulsions stabilized by β-Lg were added to the digestion cell to give final corn oil contents of 0.5, 1.0, and 2.5 wt %, and then the FFA% released versus time profiles were recorded using the pH-stat instrument.

The rate of lipid digestion decreased appreciably as the concentration of lipid present within the digestion cell increased (**Figure 3**). Again, there was a fairly good fit between the mathematical model developed under Materials and Methods and the experimental data, with the exception of the emulsion containing 0.5 wt % MCT. The rate of lipid digestion was so rapid in this system that the model tended to underestimate the %FFA released at intermediate times (2−5 min). A number of physicochemical mechanisms may explain the observed increase in digestion rate with decreasing fat content in the digestion cell: $k = 0.82, 2.66,$ and $5.12\ \mu$mol s^{-1} m^{-2} for 2.5, 1.0, and 0.5 wt % MCT, respectively. The ratio of lipase-to-lipid substrate in the digestion cell increased as the fat content decreased, which may accelerate the initial rate of lipolysis of the triacylglycerol molecules because there may have been more lipase molecules
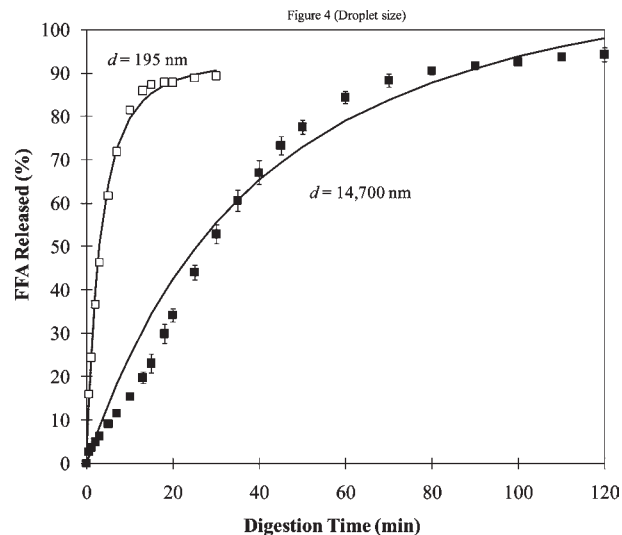
adsorbed per unit droplet surface area. In addition, the ratio of bile salts to FFA digestion products also increases as the fat content decreases, which may accelerate the lipolysis rate by helping to rapidly remove FFA digestion products from the droplet surfaces by solubilization in mixed micelles.

In reality, the human body is able to adjust the composition of the intestinal fluids to take into account differences in the amount of lipids ingested. Typically, the body would increase the concentration of lipase and bile in the small intestine in response to a higher concentration of lipids (*27*). Consequently, it may be unrealistic to keep the composition of the SSIF constant while altering the lipid content.

Effect of Droplet Size. The size of the lipid droplets reaching the small intestine varies widely depending on the nature of the ingested food and the physicochemical processes occurring within the mouth, stomach, and small intestine, for example, droplet digestion, flocculation, coalescence, and disruption (*22, 27, 28*). The size of the oil droplets within the small intestine should affect their digestion rate because the surface area of lipid exposed to the surrounding aqueous environment is inversely related to the mean droplet diameter (*4, 27, 29, 30*). Recently, it has been shown that lipid droplet size influences stomach motility and the release of gut hormones, which has important consequences for designing foods to combat obesity (*31*). It is therefore important to establish the potential influence of initial droplet size on the rate and extent of lipid digestion.

Four MCT oil-in-water emulsions stabilized by β-Lg were prepared with initial mean droplet diameters ranging from around 200 to 15000 nm (**Table 1**). The %FFA released over time was then measured for these emulsions using the pH-stat instrument. The %FFA−time profiles for the emulsions containing the smallest and largest droplets are shown in **Figure 4** along with comparisons to the predictions made using the mathematical model. The ϕ_{max} and k parameters determined by fitting the mathematical model to the experimental data for all of the emulsions are listed in **Table 1**. The rate of FFA released per unit time (FFA% s^{-1}) was appreciably higher for the emulsion with the smaller droplets (**Figure 4**), which would be expected because it has a bigger specific surface area. In other words, the surface area of lipid exposed to the surrounding aqueous phase

increases as the mean droplet diameter decreases, which provides more sites for the lipase molecules to bind (*29*). This result is consistent with earlier in vitro digestion studies that also found the rate of lipid hydrolysis (expressed per unit time) increased with decreasing droplet size (*22*, *30*).

Interestingly, when the digestion rate was normalized to the droplet surface area, it actually increased with increasing droplet size: $k = 0.65$, 0.93, 1.40, and 3.93 μmol s^{-1} m^{-2} for UFE, FE, ME, and CE, respectively (**Table 1**). The concentration of lipase within all of the emulsions was the same, and so the increase in k with increasing d_{32} may have occurred because the amount of lipase available per unit droplet surface area increased as the droplet size decreased. Thus, there may have been more lipase molecules adsorbed per unit surface area in the emulsions containing the large oil droplets than those containing smaller ones (*26*).

The above results suggest that it may be possible to control the rate and extent of lipid digestion by controlling the size of the lipid droplets within the human GI tract. Nevertheless, in vivo digestion studies in which lipid droplets were injected directly into the stomach showed that the overall extent of lipid hydrolysis was not affected by the initial droplet diameter, that is, 0.7 versus 10 μm (*28*). The differences between in vitro and in vivo experiments may be due to changes in lipid droplet size after ingestion, for example, due to droplet disruption, coalescence, or flocculation. It may therefore be important to control both the physical stability and digestibility of lipid droplets within the GI tract to control the rate of FFA release.

Effect of Initial Emulsifier Type. The droplets in oil-in-water emulsions used in the food and pharmaceutical industries may be stabilized by a variety of different emulsifiers, including small molecule surfactants, biopolymers, and phospholipids (*29*). It is therefore important to examine the potential impact of initial emulsifier type on the rate and extent of lipid digestion in emulsions. Four MCT oil-in-water emulsions stabilized by different emulsifiers (β-Lg, Tween 20, lecithin, and lyso-lecithin) were prepared with similar initial droplet diameters and concentrations (**Table 1**). The %FFA released versus time profiles were fairly similar for all of the emulsions (**Figure 5**), as were the k and ϕ_{max} parameters determined by fitting the mathematical model to the data (**Table 1**). These results suggest that initial emulsifier type does not have a large effect on the rate and extent of lipid digestion under the experimental conditions used in this study. Nevertheless, a number of previous studies have found that emulsifier type does affect lipid digestion. Wickham and co-workers found that lipid droplets initially coated by phospholipids were digested more quickly than those coated by proteins (*32*). We previously found that the resistance of lipid droplets stabilized by different emulsifiers to lipid digestion decreased in the following order: nonionic surfactant (Tween 20) > phospholipids (lecithin) > protein (caseinate or WPI) (*33*). Chu and co-workers found that the rate of lipolysis of lipid droplets could be inhibited by coating them with galactolipids with large hydrophilic head-groups that retard adsorption of bile salts and lipase through steric hindrance (*34*). Reis and co-workers found that the rate of lipid digestion was lower for droplets initially coated by monoglycerides than for those coated by proteins or phospholipids (*35*). It should be highlighted that the in vitro test conditions used in these studies were different from those used in the present study; for example, there were differences in droplet characteristics (oil type, droplet size, droplet concentration) and SSIF composition (bile salts, lipase, minerals). In our study, we tried to mimic the SSIF composition under fed conditions. Overall, these results suggest that it may be possible to retard lipid hydrolysis by altering emulsifier type,
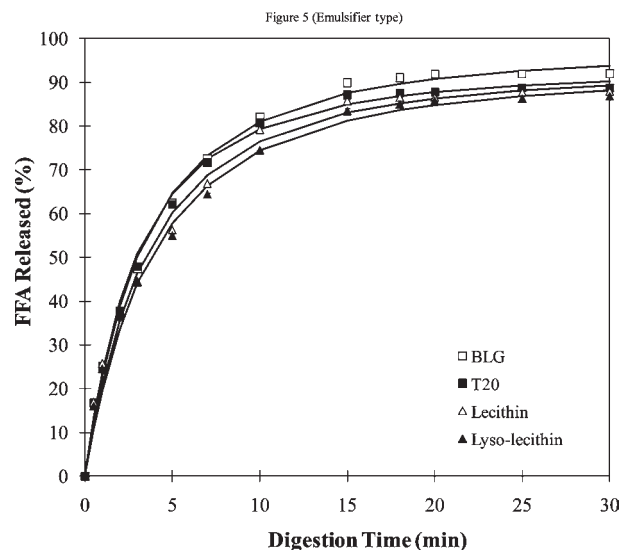

Figure 5. Influence of initial emulsifier type on the pH-stat digestion profiles of MCT oil-in-water emulsions. The curves are the best fits to the experimental data predicted using the mathematical model. Parameters derived from these curves are included in **Table 1**.

but the results depend on the conditions used in the in vitro digestion model, as well as on emulsifier characteristics such as surface activity and ability to inhibit bile salts and lipase adsorption. It should also be noted that the interfacial composition may change appreciably as lipid droplets pass through the GI tract due to digestion and competitive adsorption phenomena, for example, with bile salts, phospholipids, or FFAs (*4*, *5*). Hence, the characteristics of the droplet interfaces in the small intestine might be considerably different from those of the ingested emulsion.

Influence of Digestion on Emulsion Microstructure. The mathematical model developed in this study makes a number of assumptions that may not be valid in practice. For example, it assumes that the lipid droplets shrink as digestion proceeds and that they do not coalesce or flocculate with each other (as this would alter the specific surface area). It is therefore useful to examine changes in the microstructure of the emulsions as they pass through the pH-stat conditions to determine how well these assumptions are met.

The microstructures of 2.5 wt % oil-in-water emulsions containing droplets with different initial compositions (**Figure 6a**), sizes (**Figure 6b**), and emulsifier types (**Figure 6c**) were examined using confocal microscopy. The microstructure was recorded for (i) the initial emulsions, (ii) the initial emulsions plus SSIF (but before lipase addition), and (iii) the emulsions after 30 min of digestion by lipase. The droplets in the initial emulsions were fairly evenly distributed throughout the system. The individual droplets in the coarse MCT emulsion (CE) could easily be observed, but it was difficult to see the individual droplets in most of the emulsions containing smaller droplets because their dimensions were getting close to the resolution limit of the optical microscope. There was a distinct change in the microstructure of most of the emulsions when SSIF was added (**Figure 6**). Relatively large aggregates of lipid droplets were observed in many of the emulsions, which suggests that the droplets had become coalesced or flocculated. Droplet aggregation may have occurred because of the presence of bile salts and mineral ions in the SSIF. The bile salts may have fully or partially displaced the original emulsifier molecules from the lipid droplet surfaces, and then the cationic sodium (Na$^+$) and calcium (Ca^{2+}) ions may have promoted

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J. Agric. Food Chem., Vol. 58, No. 13, 2010 **8091**
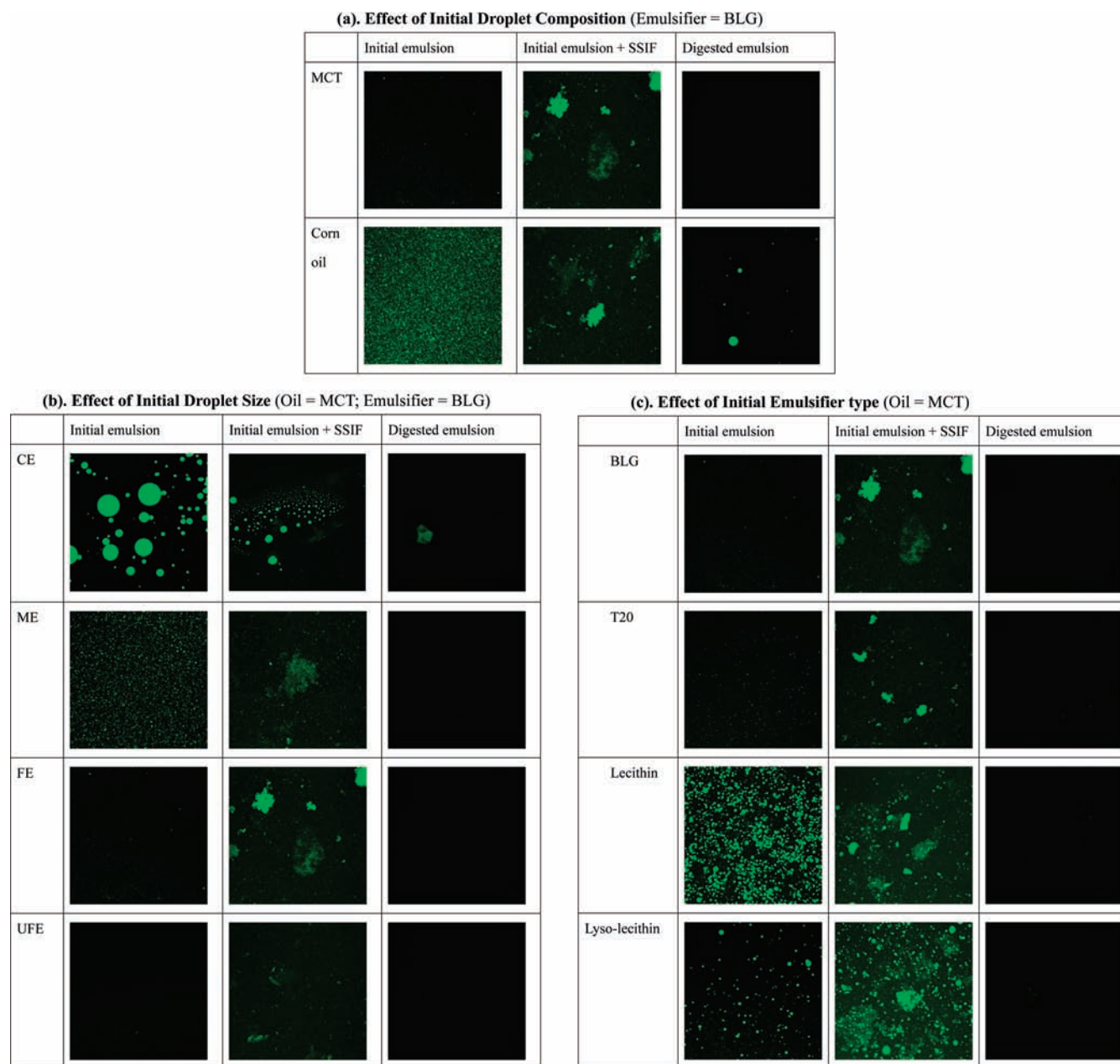

Figure 6. Confocal microscopy images of 2.5 wt % oil-in-water emulsions. The microstructure was recorded for (i) initial emulsions, (ii) initial emulsions plus SSIF (but before lipase addition), and (iii) emulsions after 30 min of digestion by lipase. See **Table 1** for additional information on composition and properties.

droplet flocculation through charge neutralization and bridging effects (*29*). The fact that the droplets were flocculated in the emulsions might alter their rate of lipid digestion, because it would be more difficult for lipase molecules to penetrate through a floc and reach the lipid droplets in the interior. Droplet flocculation would therefore be expected to decrease the effective surface area of lipid exposed to the surrounding aqueous phase. The magnitude of this effect is likely to depend on the nature of the flocs formed, for example, the strength of the forces holding the droplets together and the floc dimensions. This effect is difficult to include in the mathematical model because it is highly system dependent. Nevertheless, the values of ϕ_{max} and k determined using the model should reflect these effects; for example, if flocculation occurred, k may be less than expected for a non-flocculated system. In this case, the model developed in this work would still be a useful means of comparing treatments. After 30 min of digestion, there was no visual evidence of any lipid droplets remaining in most of the emulsions, indicating that lipid

digestion was complete, which supported the pH-stat measurements (**Figures 2−5**). The only exceptions were the corn oil emulsion (**Figure 6a**) and the MCT emulsion containing the largest droplets (**Figure 6b**), where some lipid droplets were still observed after 30 min of digestion. These results are again consistent with the pH-stat measurements because neither the corn oil emulsions (**Figure 2**) nor the coarse MCT emulsions (**Figure 4**) were fully digested within 30 min. Ideally, it would be useful to measure the change in the particle size distribution of the emulsions during the digestion process to establish if the droplets did shrink, but this is analytically difficult because of the presence of various other components in the digestion cell that scatter light and the rapid nature of the digestion process.

Conclusions. In vitro digestion models are being used increasingly in the food and pharmaceutical industries to screen the digestibility of products with different compositions and structures. We have developed a new mathematical model that can be used to interpret the free fatty acid versus time profiles generated

by pH-stat methods. This model can be used to quantify the impact of system composition and structure on the rate (k) and extent (ϕ_{max}) of lipid digestion. In this case, the rate is the amount of free fatty acids generated per unit time per unit surface area of oil. We found that the rate of lipid digestion (k) increased with decreasing molecular weight of fatty acids in the oil phase, decreasing lipid droplet concentration, and increasing droplet size. These effects are interpreted in terms of the concentrations of various components (e.g., lipase, bile, and FFA) involved in the digestion reaction relative to the available droplet surface area. This study has important implications for designing and testing delivery systems that control lipid digestion.

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