Hydrolysis of emulsions with different triglycerides and droplet sizes by gastric lipase in vitro. Effect on pancreatic lipase activity

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Digestion of dietary fat first takes place in the stomach in numerous species including humans. Thus, we have studied in vitro the gastric lipase-catalyzed hydrolysis of four emulsions devoted to tube feeding. The emulsions contained phospholipids, sugar-esters, and triglycerides in the form of either medium-chain triglycerides (MCT) or long-chain triglycerides (LCT) or a ¼ (wt/wt) mixture of both (MCT/LCT). The mean droplet sizes were 0.19 μm (MCT), 0.43 μm (LCT), and 0.46 μm or 3.18 μm (MCT/LCT). Gastric lipase activity was greater on the fine mixed emulsion than on the coarse one, but enzyme affinities and bindings onto droplets were comparable. The affinity of gastric lipase was higher for LCT emulsion. Free fatty acid concentration played a key role in the progressive inhibition of lipolysis, the extent of which was dependent on the emulsion surface area. Pre-hydrolyzing emulsions by gastric lipase helped pancreatic lipase binding to the fine droplets and enhanced the subsequent activity of pancreatic enzyme. Relevant implications of nutritional importance can be drawn concerning lipolysis in the stomach, such as (1) suitability of mixed emulsions, (2) key role of the nature of triglycerides, (3) apparent advantage of small droplet size (0.4 versus 3 μm), and (4) potential detrimental effect of free fatty acids present in emulsions to be tube fed in the stomach, especially in patients with reduced pancreas capacity.
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Introduction

In humans, the digestion and assimilation of dietary triglycerides is governed by two key enzymes: a pre-duodenal lipase of gastric origin[1,2] and a pancreatic lipase.[3,4] It is now well recognized that the first step of dietary fat digestion takes place in the stomach, where gastric lipase is secreted and hydrolyzes, under acidic conditions, 10 to 60% of ingested fats.[5] In fact, in human adults, the total activity of lipase present in the stomach mucosa amounts to about 20% of that present in the normal pancreatic gland.[2] From recent studies,[5,6] it has been concluded that the gastric enzyme continues to hydrolyze dietary fat in the duodenum, especially in newborn infants or in pathological pancreatic insufficiencies. This may explain why about 70% of dietary fat continues to be absorbed in patients lacking pancreatic lipase, which is considered a key enzyme in the normal digestion process.[4] The potential role of gastric lipase is reinforced by our finding that pre-duodenal lipases readily adapt to the level of dietary fat, as recently demonstrated for lingual lipase in the rat[7] and gastric lipase in the rabbit[8] and pig.[9]

Several factors are already known from in vitro studies to alter the activity of gastric or lingual lipases. Above all, the characteristic feature of gastric and pancreatic lipases is their specificity of action on insoluble emulsified substrates. Thus, the surface concentration and composition and the physico-chemical properties of the lipid/water interface play a key role; first in enzyme adsorption onto the particle surface, then catalyzing the removal of fatty acids from triglyceride molecules. Without consideration of the emulsion droplet sizes, the hydrolytic activity of gastric lipase was previously measured on pure short

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chain,[10,11] medium chain (MCT),[12] and long chain triglycerides (LCT).[10,12,13]

The concerted action of gastrointestinal lipolytic enzymes to ensure fat digestion has been investigated recently. Some of these in vitro[13,14,15,16] or in vivo[17,18] studies have suggested that gastric lipase might promote the hydrolysis of dietary LCT by pancreatic lipase, but the mechanisms involved are not yet understood. Free fatty acids, which can be generated by the partial lipolysis occurring under acidic conditions, have been implicated in this process.[13,15,16,19,20] These observations could have important implications under the physiological conditions, because experimentally suppressing the first lipolysis step in the stomach leads to a significantly lower assimilation of dietary fat in laboratory animals.[17,18]

In a previous study[14] we examined in vitro the action of pancreatic lipase on complex emulsions. Such emulsions are increasingly used for enteral feeding to meet both technical constraints and nutritional requirements. In the present study, we have determined the activity of gastric lipase on comparable emulsions. Our hypothesis is that the activity of gastric lipase is dependent on droplet size and/or composition of the substrate emulsion. Therefore, our objective was to determine the effect of size (from 0.19 to 3.18 μm) or triglyceride composition (pure MCT or LCT, mixed MCT/LCT) of emulsified substrate droplets on the activity of gastric lipase. Moreover, we investigated the effect of free fatty acids on the bindings and activities of gastric and pancreatic lipases on the mixed MCT/LCT emulsions with different droplet sizes.

Methods and materials

Emulsions

The four emulsions used—MCT, LCT, and mixed MCT/LCT with 1:4 weight ratios (0.46 μm or 3.18 μm mean droplet sizes)—came from Cernep-Clintec Technologies (Le Plessis-Robinson, France). IVELIP, a commercial emulsion suitable for parenteral nutrition (Cernep-Clintec Technologies) was used as a reference in two experiments. It basically contained (g/100 g) soybean oil (20), egg lecithin (1.2), sodium oleate (0.03), and glycerol (2.5). Its mean droplet size was 0.44 μm. The four other emulsions used were prepared by using a high pressure homogenizer (APV Baker, Evreux, France). The chemical compositions and some physico-chemical parameters of the emulsions are given in *Table 1*. These emulsions were complex mixtures made to satisfy nutritional requirements. Triglycerides were either MCT containing (g/100 g fatty acids) C8 : 0 (48.5), and C10 : 0 (39.7), or LCT essentially provided by various vegetable oils. The fatty acid composition of the LCT mixture was (g/100 g fatty acids): C16 (11.5), C18 (82.6), C20 (2.9), and C22 (2.5), with 54% monounsaturated and 23% polyunsaturated fatty acids. Mixed MCT/LCT emulsions contained (g/100 g triglycerides): MCT (20) and LCT (80) or (mol/100 mol) about MCT (34) and LCT (66). Soybean lecithins and sugar-esters (non-ionic sucrose fatty acid esters, widely used as emulsifying agents) were used as emulsifiers *(Table 1)*.

The distribution of emulsion droplet sizes was determined by using a particle size analyzer Capa-700 (Horiba, Kyoto, Japan) as previously described.[14] Mean size values were calculated by the particle-sizer software from the droplet size distribution expressed as a fraction of total droplet volume. Pure MCT, LCT, and fine MCT/LCT emulsions showed a narrow distribution, expressed in fractions of total particle volume or area. Up to 90% of the area was represented by 0.05 to 0.25 μm, 0.05 to 0.45 μm, and 0.05 to 0.65 μm droplets for the MCT, LCT, and fine MCT/LCT emulsions, respectively. The mean size of the pure MCT emulsion was slightly lower (0.19 μm) than that of pure LCT (0.43 μm) or fine MCT/LCT emulsion (0.46 μm) because of the differences in the physico-chemical properties of the emulsions made with MCT only. The coarse MCT/LCT emulsion (3.18 μm) showed a wider size spectrum, though more than half of the area was represented by 1.5 μm to 4.5 μm droplets. From the distribution of particle size classes obtained for a given emulsion, the particle-sizer software calculated the specific interfacial area (Sw) exhibited, from Equation 1.

$$Sw = 6/dx \sum_{i=1}^{n} (F'_i/D_i) \qquad (1)$$

with d: particle density and F'_i: fraction of particles with a given diameter D_i. Sw is expressed in cm^2/g of lipid particle and the interfacial area concentration (cm^2/L) in the assays was calculated from the lipid concentration (g/L).

Enzymes

Human gastric juice was obtained from gastric aspirates collected for diagnostic purposes in adult patients under pentagastrin stimulation. They were generous gifts from Drs. J. Peyrot and J. Salducci (Gastroenterology Department, Hospital Nord, Marseille, France). Samples with comparable high activities were neutralized, pooled, and stored at −70° C. All experiments were thus done with the same enzymatic source. The lipase activity and the specific activity of the pooled samples were 48,000 units/

Table 1 Compositions and some physico-chemical parameters of emulsions

Component (g/L)	Emulsions			
	Coarse MCT/LCT	Fine MCT/LCT	MCT	LCT
Medium-chain triglycerides	40.0	40.0	200.0	0.00
Long-chain triglycerides (mixed oils)	160.0	160.0	0.0	200.0
Emulsifiers (soybean lecithin, sugar-esters)	42.3	42.3	42.3	42.3
Physico-chemical parameters				
Median diameter (μm)[a]	3.18	0.46	0.19	0.43
pH	6.7	6.7	6.6	6.5
Osmolality (mmol/kg)	6	6	3	7
Conductivity (μS/cm)	430	435	428	454

[a]Mean diameters were calculated as reported[14] from duplicate meaurements performed by the particle-sizer software (Capa 700, Horiba).

L and 25.2 units/mg protein, respectively, with tributyrin as the substrate.[21]

Gastric lipase was purified to homogeneity from rabbit gastric mucosa as previously described by Moreau et al.[22] Minor modifications were made such as using an S-sepharose fast flow (Pharmacia, Uppsala, Sweden) cation exchange chromatography instead of a fast protein liquid chromatography system using a mono S column. The specific activity of the preparation was 450 units/mg of protein with tributyrin as the substrate.

Purified porcine pancreatic lipase (specific activity: 1370 units/mg of protein) and porcine pancreatic colipase (specific activity: 1590 units/mg of protein) were purchased from Boehringer Mannheim (Mannheim, Germany).

Lipid analysis

The phase partition of phospholipids present in the emulsions, i.e., the distribution of phospholipids between the triglyceride droplets (cream) and the aqueous phase (infranatant) was determined. The ultracentrifugation conditions were adapted to each individual emulsion in the presence of water or the reaction medium (pH 5.40) to prevent the emulsion from breaking up (absence of an oil phase overlayered on the cream). About 97 to 99% of the triglycerides were found in the cream phase and therefore, under the various ultracentrifugation conditions used, there was no significant contamination of the infranatant aqueous phase with triglyceride molecules (data not shown). One mL of each emulsion was mixed with 14 mL distilled water or with gastric lipase medium (pH 5.40) that contained: 30 μmol/L bovine serum albumin, 6 mmol/L $CaCl_2$, 150 mmol/L NaCl. The mixture was mechanically stirred for 5 min; 7 mL of the mixture was put in a polyallomer centrifuge tube and overlayered with 1 mL of distilled water. Samples were centrifuged (from 28,000*g* to 160,000*g* and for 4 min to 22 hr) in an SW 40 ti rotor at 22° C in a Beckman L 2-65B ultracentrifuge (Beckman Instruments, Palo Alto, CA USA). The centrifuge was operated without braking. The infranatants were collected by slicing the polyallomer tubes. Lipids were extracted using the method of Folch et al.[23] Phosphorus was measured after mineralization[24] and triglycerides were assayed by using an enzymatic method[25] in the form of PAP 150 assay kit (BioMerieux, Marcy-l'Etoile, France).

Gastric and pancreatic lipase activity measurements

Human gastric lipase activity was routinely measured with a pH-stat titrator (Metrohm, Herisau, Switzerland) as previously described[10,11,21] Human gastric lipase reactions were carried out at 37° C and pH 5.40. The enzyme was incubated for 1 min to measure the initial velocity as previously described[10,21] unless otherwise stated, because longer reaction times decrease lipase activity. The reaction medium was a 15 mL mixture containing 150 mmol/L NaCl, 6 mmol/L $CaCl_2$, and 30 μmol/L fatty acid-free bovine serum albumin (Sigma, La Verpilliere, France) to prevent interfacial denaturation of the enzyme as previously described[10] and 2 or 4 mL of each emulsion. For each assay, 200 or 400 μL of gastric juice was used. At the end of the reaction time, the liberated fatty acids were fully titrated at pH 9.40 with 0.02 mmol/L NaOH. The time required to adjust from pH 5.40 to pH 9.40 was short and reproducible (5 to 7 sec) as in previous studies,[10,21] and the activity exhibited by gastric lipase at this final pH was negligible. Controls without enzyme were run in parallel and this value was subtracted from the assay for each lipase measurement. The activity of pure rabbit gastric lipase (used for V_m and K_m determinations) was measured following the same procedure, but the reaction was carried out at pH 4.00, which is the optimal pH in this case.[21] For each assay, 22 μg of pure lipase was added. The two gastric lipases, from either human or rabbit, have been reported to have close physiological, molecular, and kinetic properties.[10,21,22]

Porcine pancreatic lipase activity was measured potentiometrically with a pH-stat titrator.[26] The molar ratio between lipase (25 nmol/L) and colipase (50 nmol/L) was within the physiological range and was kept constant. The reaction medium was a 15 mL mixture containing 2 mmol/L Tris-Hcl, 150 mmol/L NaCl, 0.5 mmol/L $CaCl_2$, 8 mmol/L mixed bile salts (glycocholate : 2.32 mmol/L, glycodeoxycholate : 3.2 mmol/L, taurocholate : 0.64 mmol/L, taurodeoxycholate : 1.84 mmol/L) (Calbiochem, San Diego, CA USA), 2 mL emulsion. The pancreatic lipase activity was recorded at pH 7.50 until the maximal enzyme velocity was reached. The time needed to obtain linear steady-state recording was defined as the lag time.[19] The fatty acids released at pH 7.50 under lipolysis were titrated at the end of the reaction at pH 9.50. In some experiments, various amounts of oleic acid and caprylic acid were added to the assay medium prior to enzyme addition to give final concentrations ranging from 0–10 mmol/L.

To study the concerted action of gastric and pancreatic lipases, the emulsions were first submitted to hydrolysis by gastric lipase (400 μL human gastric juice) at pH 5.40, for 0, 1, 2, 8, or 16 min (or up to 120 min in one experiment), as described above. At the end of the reaction, 1 mL of a stock buffer solution (pH 7.50) containing the mixture of bile salts was added to the assay medium (final concentration: 8 mmol/L bile salts and 2 mmol/L Tris-HCl). The pH of the reaction medium was quickly adjusted (5 to 10 sec) to 7.50 with NaOH.[15] Pancreatic colipase and lipase were added and the activity of pancreatic lipase was measured as described above. For both enzymes, one lipase unit was defined as 1 μmol fatty acid liberated per minute.

Gastric and pancreatic lipase binding measurements

The method previously developed by Borgström et al.[19] for pancreatic lipase binding measurements was used in both cases. To measure the fraction of gastric lipase bound to the emulsions as a function of incubation time, 2 mL of emulsion was diluted with 12 mL of a solution containing 150 mmol/L NaCl, 0.5 mmol/L $CaCl_2$, pH 5.40. Then, human gastric juice (400 μL) was added to the mixture and lipolysis took place at a constant pH (pH-stat titrator) for different periods of time, i.e., 30 sec, 2 min, or 5 min. To stop the enzymatic reaction, the mixture was put into test tubes containing 2 mL of ice-cold buffer solution. Emulsion droplets were floated by ultracentrifugation at 66,000*g* for 15 min and at 4° C, in an SW 40 Ti Beckman rotor. The infranatants were collected and the activity of gastric lipase present was measured. Controls were done by replacing emulsions with distilled water. The fraction of gastric lipase not present in the subphase in the presence of emulsion was considered to be bound to the droplet surface.[13,19]

After sequential hydrolysis of the emulsions as described above (first by gastric lipase for 0 or 16 min, and secondly, by pancreatic lipase for 30 sec), we used the procedure described above for gastric lipase to perform the measurement of the binding of pancreatic lipase to the emulsions. Binding measurements were performed in quadruplicate.

Statistics

All experiments were done with four replicates and the results are expressed as means ± SE of four values. The statistical significance of the differences observed was assessed by one-way analysis of variance (ANOVA) and Fisher's test ($P < 0.05$) or by Student's *t* test ($P < 0.05$), by using the Statview II micro-computer program (Abacus, Berkeley, CA USA).

Results

Phase partitioning of phospholipids

After incubation in distilled water *(Figure 1A)*, the proportion of phospholipids in the infranatant was inversely and significantly related to the size of the emulsions : 33% of the phospholipids were in the infranatant for the smallest emulsion (MCT, 0.19 μm) and 78.4% of the phospholipids were in the infranatant for the largest emulsion (MCT/LCT 3.18 μm). The fraction of phospholipids present in the infranatant with the IVELIP emulsion (used as a control) was significantly lower than those of emulsions with comparable size. In fact, IVELIP contained only one emulsifier (egg lecithins, 1.20%) as compared with soybean lecithins and sugar-esters (4.30%) in the other emulsions.

Incubation in the gastric lipase medium (pH 5.40) did not induce marked changes in the mean droplet sizes (data not shown). The phospholipid partitioning toward the aqueous phases *(Figure 1B)* at acidic pH was slightly but significantly decreased in the case of the MCT and LCT emulsions and did not markedly change in the case of both mixed MCT/LCT emulsions. An opposite trend was given by IVELIP.

Gastric lipase kinetics

The enzyme activity measured on either the fine or the coarse MCT/LCT emulsion exhibited bell-shaped curves between pH 3.00 and 7.00. The maximal activities were reached around pH 5.00 to 5.40 for both mixed MCT/LCT emulsions. The pH profile of human gastric lipase activity slightly depended on the particle size of the triglyceride emulsions used. At pH 3.00, gastric lipase still retained 60% of its maximal activity with the fine MCT/LCT emulsion as substrate but exhibited only 30% of its maximal activity when the substrate was the coarse MCT/LCT emulsion. Comparable differences were observed at pH 6.00. The maximal activities measured on the pure MCT or LCT emulsions were also in the pH range 5.00–5.40.

The activity of pure rabbit lipase *(Figure 2 A, B)* on the

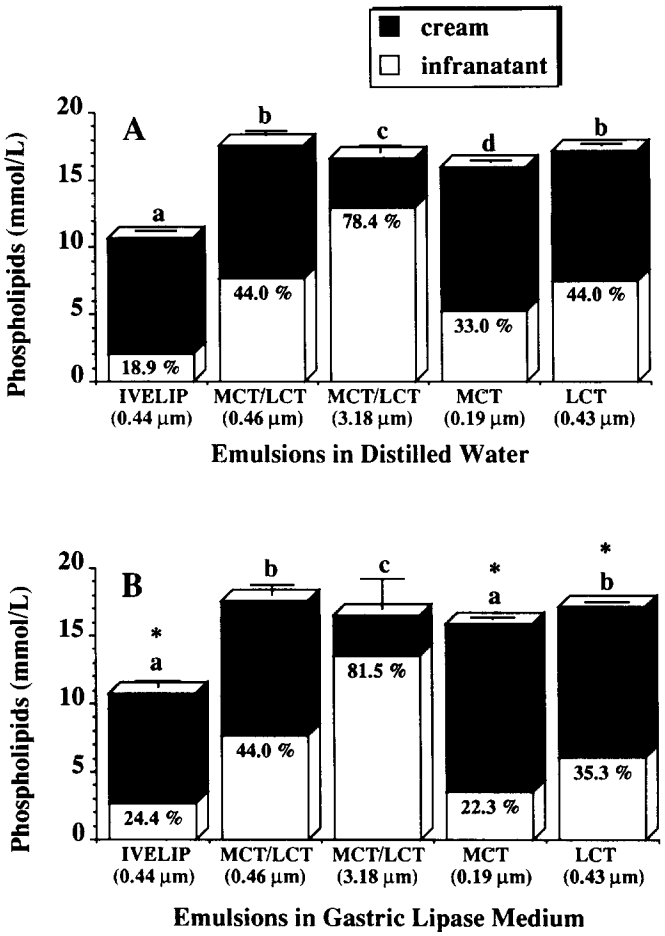

Figure 1 Partitioning of phospholipids between the emulsion phase (cream) and the soluble phase (infranatant). A: phospholipid distribution after incubation in distilled water. B: phospholipid distribution after incubation in gastric lipase medium (30 μmol/L bovine serum albumine, 6 mmol/L $CaCl_2$, 150 mmol/L NaCl, pH 5.40). IVELIP was used as a reference only. For details, see Methods and materials. Each value represents the mean ± SE of four measurements. Significant differences (ANOVA, $P < 0.05$) in phospholipid partitioning for a given incubation condition are noted by different letters (a–d). The presence of an asterisk in *Figure 1B* indicates a significant difference (Student's *t* test, $P < 0.05$) in phospholipid partitioning between the two incubation conditions for a given emulsion.

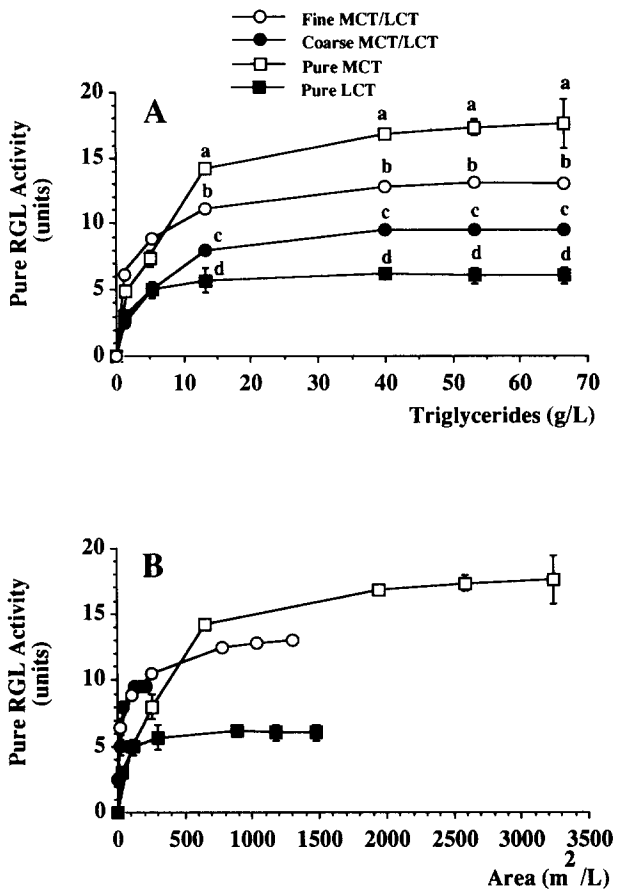

Figure 2 Effect of substrate concentrations (A) and emulsion area concentrations (B) on the lipolytic activity of pure rabbit gastric lipase (RGL). The incubation mixture (15 mL) consisted of the indicated amount of substrate dispersed in the gastric lipase medium and 22 μg of pure RGL. The reaction was performed at 37° C and pH 4.0. Each point represents the mean ± SE of four replicates (error bars smaller than the point width cannot be shown). Significant differences (ANOVA, $P < 0.05$) between emulsions at given triglyceride concentrations are noted by different letters (a–d).

fine MCT/LCT (0.46 μm) and the coarse MCT/LCT (3.18 μm) emulsions showed different patterns when the amount of substrate (g/L or m^2/L) increased, with a significantly higher V_{max} (+ 18.6%) obtained with the fine MCT/LCT emulsion *(Table 2)*. As shown in *Figure 2*, pure gastric lipase exhibited the highest maximal velocity on the pure MCT emulsion and the lowest on the pure LCT emulsion. This discrepancy was clearly shown by the different V_{max} calculated from the linear Lineweaver-Burk plots *(Table 2)*. With human gastric lipase (gastric juice), the highest maximal enzyme velocity was obtained with the pure MCT emulsion (about 16 units). A much lower velocity was measured with the pure LCT emulsion (about 5 units) and intermediate values were recorded (about 8 units) with the two mixed MCT/LCT emulsions.

As calculated from the Lineweaver-Burk plots obtained with pure rabbit gastric lipase and six or seven substrate concentrations, the apparent K_m for the pure MCT emulsion was five-fold higher than that determined for the pure LCT emulsion when expressed as substrate concentration, i.e., g/L triglycerides *(Table 2)*. In fact, the true representation of the substrate concentration in such heterogeneous systems is the substrate interface concentration, i.e., m^2/L, as previously suggested.[27] Under these latter conditions *(Table 2)*, the apparent K_m in the presence of the pure MCT emulsion became much higher (× 10.3) than that measured with the pure LCT emulsion. When expressed in m^2/L, the apparent K_m values were not significantly different either with the coarse mixed MCT/LCT, the fine mixed MCT/LCT, or the pure LCT emulsion. Thus, no marked difference in the affinity was observed when the mean particle size of the emulsions varied from 0.43 to 3.18 μm.

The amount of free fatty acid released by human gastric lipase acting at pH 5.40 on various emulsions was determined as a function of time, from 30 sec to 30 min. As shown in *Figure 3*, the rate of lipase hydrolysis decreased with time with all emulsions used. The average loss of lipase activity (per min) after a 30 min incubation time period, as compared with 30 sec, was in the range of 61.2–80.6% for the four emulsions used. As expected from kinetic data *(Figure 2)*, the amounts of free fatty acid released after 5 and 30 min were significantly higher with the pure MCT emulsion. The amounts of free fatty acid released by the two mixed MCT/LCT emulsions were slightly but significantly different *(Figure 3)*.

The role of generated endogenous free fatty acids in the inhibition of gastric lipase activity was investigated by externally adding some relevant fatty acids, i.e., caprylic acid (C8:0) and oleic acid (C18:1). As shown in *Figure 4*, increasing the caprylic acid concentration from 0 to 10 mmol/L significantly decreased the initial velocity of human gastric lipase on the mixed MCT/LCT emulsions. Above 5 mmol/L, the enzyme activity on the coarse emulsion was almost completely abolished. Up to 10 mmol/L of oleic acid did not alter the gastric lipase activity on the fine mixed emulsion. With the coarse mixed emulsion, oleic acid had no effect below 5 mmol/L, but above this concentration it markedly inhibited the enzyme activity.

Gastric lipase binding

To study the mechanisms involved in this inhibition process, the extent of binding of gastric lipase onto emulsified particles was measured after various times of lipolytic reaction (as in *Figure 3*). At the beginning of the reaction (0.5 min), almost all the gastric lipase present was bound to the emulsified droplets, with either the fine or the coarse mixed MCT/LCT emulsion *(Table 3)*. After 2 or 5 min of reaction, this figure was unchanged with the fine emulsion, but slightly decreased with the coarse emulsion. After 5-min incubation, the extent of binding of gastric lipase was slightly but significantly lower on the coarse than on the fine mixed emulsion.

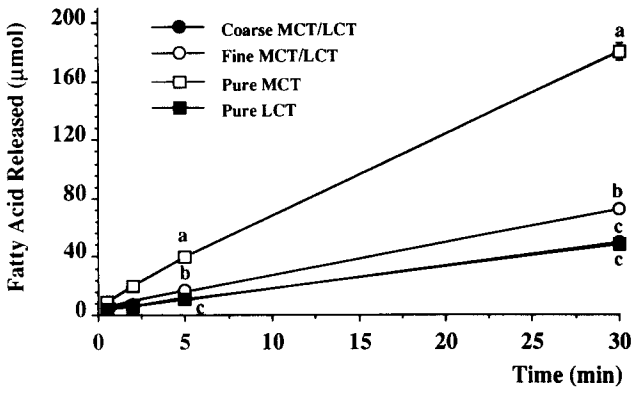

Figure 3 Amount of free fatty acids released by human gastric lipase as a function of incubation time. The enzyme (200 μL human gastric juice) was incubated at 37° C in a medium (15 mL) containing 4 mL emulsion at pH 5.40. Free fatty acids were titrated at pH 9.40. Each point represents the mean ± SE of four replicates. Significant differences (ANOVA, $P < 0.05$) between emulsions at given time points (above 2.5 min) are noted by different letters (a–c).

Table 2 Kinetic parameters of pure rabbit gastric lipase with different emulsified substrates[a]

Emulsions	apparent K_m (g/L)	apparent K_m (m^2/L)	V_{max} (μmol FFA/min)[b]
Coarse MCT/LCT (3.18 μm)	4.3 ± 0.7[a]	13.7 ± 2.3[a]	10.2 ± 0.8[a]
Fine MCT/LCT (0.46 μm)	1.3 ± 0.3[b]	24.8 ± 5.9[a]	12.1 ± 0.4[b]
MCT (0.19 μm)	6.2 ± 0.5[c]	305.2 ± 26.6[b]	17.9 ± 0.2[c]
LCT (0.43 μm)	1.3 ± 0.4[b]	29.7 ± 9.7[a]	6.0 ± 0.3[d]

[a]The apparent K_m and V_{max} values, given as the means ± SE of four measurements, were obtained from linear (1/v f 1/s) Lineweaver-Burk plots derived from the experimental data showed in *Figure 2*.
[b]FFA, free fatty acid.
The presence of different letters[a–d] indicates a significant difference between emulsions (ANOVA, $P < 0.05$).

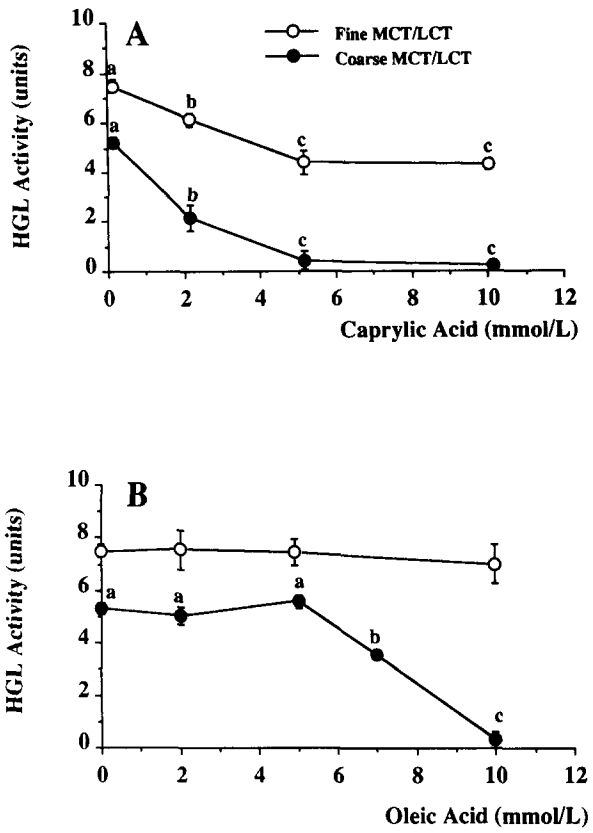

Figure 4 Effects of adding increasing amounts of free fatty acids such as (A) caprylic acid and (B) oleic acid, on human gastric lipase (HGL) activity hydrolysis. The enzyme (200 μL human gastric juice) was incubated at 37° C in the assay medium containing 2 mL emulsion. The reaction was performed at pH 5.40. Each value represents the mean ± SE of four replicates. Significant differences (ANOVA, $P < 0.05$) between emulsions at given exogenous free fatty acid concentrations are noted by different letters (a–c).

Gastric lipase pre-hydrolysis and activity of pancreatic lipase

The activity of pancreatic lipase on the fine or the coarse mixed MCT/LCT emulsion was measured as a function of the extent of pre-hydrolysis by gastric lipase *(Figure 5A)*. The pre-hydrolysis of emulsions by gastric lipase for 1 to 2 min slightly but significantly increased the maximal velocity of pancreatic lipase. Longer incubation times of up to 16 min led to only slightly enhanced enzyme activity. Conversely *(Figure 5B)*, the lag time, i.e., the time needed to reach maximal steady-state pancreatic lipase activity, was markedly and significantly reduced by pre-hydrolyzing the emulsions with gastric lipase and was almost suppressed after a 16-min pre-incubation period. It is noteworthy that without pre-hydrolysis, the pancreatic lipase lag time was two-fold longer with the fine mixed emulsion than with the coarse one (2.23 versus 0.91 min).

As shown in the insert *(Figure 5B)*, the lag time of pancreatic lipase decreased as a function of the amount of free fatty acids released during gastric lipase pre-hydrolysis. To get the lag time reduced to a negligible value (0.3 min), the free fatty acid bulk concentrations were 6.2 and 1.2 mmol/L, respectively, with the fine and the coarse emulsion. Consequently, the calculated free fatty acid bulk concentration ratio (5.2) was close to that of the surface area ratio (6.1) derived from the two emulsions used. Under these conditions, comparable interfacial accumulations of free fatty acids were obtained i.e., 11.9 and 14.2 μmoles/m² available surface area, with the fine and the coarse emulsion, respectively.

The influence of added exogenous free fatty acids was investigated as shown in *Figure 6*. Adding up to 10 mmol/L of caprylic acid did not affect the activity of pancreatic lipase on either the fine or coarse mixed MCT/LCT emulsions *(Figure 6A)*. Increasing the concentration of oleic acid *(Figure 6B)* slightly decreased (−23%) the activity of pancreatic lipase above 2 mmol/L on the fine emulsion, whereas

Table 3 Lipase binding to emulsion droplets

	% bound Gastric lipase	
Incubation time (min)	Coarse MCT/LCT emulsion (3.18 μm)	Fine MCT/LCT emulsion (0.46 μm)
0.50	88.1 ± 3.2	86.6 ± 3.6
2.00	79.0 ± 3.7	85.5 ± 1.1
5.00	71.6 ± 0.3	89.9 ± 0.9*
	% bound pancreatic lipase	
Human gastric lipase pre-incubation time (min)	Coarse MCT/LCT emulsion (3.18 μm)	Fine MCT/LCT emulsion (0.46 μm)
0	95.9 ± 0.8	47.6 ± 1.6^{x*}
16	94.2 ± 1.3	82.5 ± 0.7^{y*}

The measurements of the binding of human gastric lipase (HGL) on coarse and fine emulsions as a function of incubation time and the binding of porcine pancreatic lipase as a function of pre-incubation time with HGL were performed as described in details in Methods and materials. Values are expressed as means ± SE of four measurements.
The presence of different letters (^{x,y}) indicates a significant difference between incubation times for a given emulsion and enzyme (ANOVA, $P < 0.05$).
*Significant differences between emulsions for a given time and enzyme (Student's t test, $P < 0.05$).

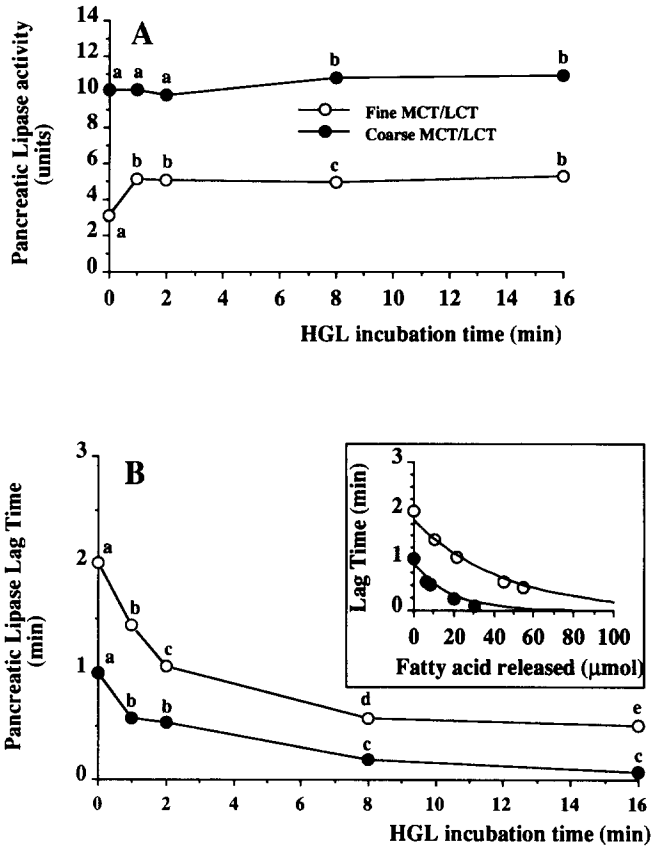

Figure 5 Effect of pre-hydrolysis by human gastric lipase (HGL) on (A) porcine pancreatic lipase activity and (B) enzyme lag time. HGL (400 μL) was added at the beginning of the experiment in a reaction medium containing 150 mmol/L NaCl, 0.5 mmol/L CaCl2, 30 μmol/L bovine serum albumin, 2 mL emulsion, pH 5.40 at 37° C. After different incubation times with gastric lipase, Tris-HCl buffer containing a mixture of bile salts was added and the pH of the incubation mixture was raised to 7.50. At that time, porcine pancreatic colipase and lipase (molar lipase:colipase ratio 1:2) were added and lipase activity recorded. Insert: plot of the pancreatic lipase lag time against the concentration of free fatty acids released during hydrolysis by HGL. Each value represents the mean ± SE of four replicates. Significant differences (ANOVA, $P < 0.05$) between HGL incubation times for a given emulsion are noted by different letters (a–e). Significant differences (Student's t test, $P < 0.05$) were found between emulsions for every HGL incubation time in *Figures 5A and 5B* (not shown on the graph).

the extent of inhibition steadily increased (10 mmol/L) with the coarse emulsion (−69%).

The extent of binding of pancreatic lipase on the emulsified droplets was measured without or after pre-hydrolyzing the emulsions by gastric lipase for 16 min *(Table 3)*. The results show that almost all the pancreatic lipase present was bound to the droplets of the coarse emulsion with no significant influence of pre-hydrolysis. With the fine emulsion, the fraction of enzyme bound to the droplets (about half of the enzyme present) was significantly lower without pre-hydrolysis. The 16-min pre-hydrolysis by gastric lipase (conditions that suppressed lag time, *Figure 5B*) led to a significant increase in the binding of pancreatic lipase onto the fine emulsion. This extent was slightly but significantly lower than that measured on the coarse mixed emulsion.

Discussion

In numerous patients, some key steps involved in fat digestion and absorption are damaged, leading to fat malabsorption, reduced energy supply, and health complications. Thus, it becomes particularly important to take into account the importance of gastric lipase as the first key enzyme involved in fat digestion, especially in situations in which the pancreatic lipase levels or bile secretion are severely impaired.

To optimize the efficiency of fat digestion, it seems essential to determine what are the key factors governing triglyceride lipolysis either under the acidic conditions in the stomach or the neutral medium in the small intestine. In the context of tube feeding, complex and stable emulsified mixtures are needed, both for technological reasons (increased shelf-life) or nutritional needs (fulfilling various nutrient requirements). This explains why complex emulsified mixtures were used in the present study, as compared with the much simpler ones generally used in in vitro studies.[10,12,27,28] These complex emulsions are characterized by the presence of excess phospholipids, as observed in the case of emulsions used for parenteral nutrition, such as INTRALIPID (Kabi-Vitrum, Paris) or herein IVELIP. The amount of excess phospholipids depends on the total amount added and the sizes of the emulsified droplets.[29] Thus, as expected, we

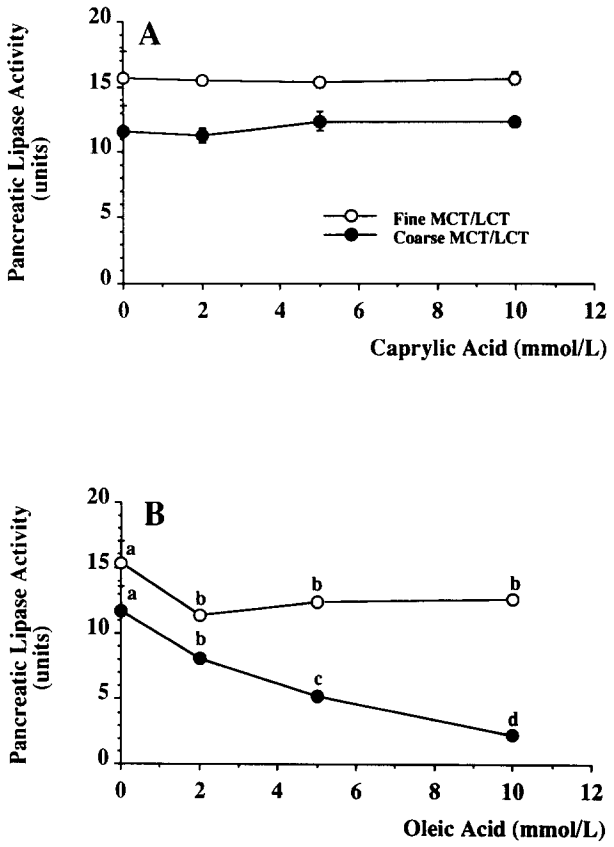

Figure 6 Effect of adding increasing amounts of caprylic acid (A) and oleic acid (B) on porcine pancreatic lipase activity (molar lipase:colipase ratio 1:5). The reaction was performed at 37° C and pH 7.50. Each value represents the mean ± SE of four replicates. Significant differences (ANOVA, $P < 0.05$) between emulsions at given exogenous free fatty acid concentrations are noted by different letters (a–c).

observed *(Figure 1)* that under neutral or acidic conditions, excess of phospholipids was lowest in the case of the MCT emulsion (mean particle diameter: 0.19 μm) and highest for the MCT/LCT emulsion (mean particle diameter of 3.18 μm). The behavior of sugar-esters was not investigated. The phospholipid concentrations in the aqueous phase ranged between 3.3–5.4 mmol/L. Increasing the lecithin concentration 10 fold (2 versus 0.2 mmol/L) or from 0 to 5 mmol/L had no marked effect on the hydrolysis of a triglyceride emulsion by rat lingual lipase[12] or human gastric lipase,[11] respectively. Thus, it seems very likely that variations in the phospholipid concentration in the aqueous phase cannot account for variations of human gastric lipase activity on the various emulsions studied.

The emulsions tested were stable under acidic conditions (pH 4.0 to 5.4), with no significant shift in the droplet sizes. The optimum pH for human gastric lipase activity measured with pure or mixed MCT/LCT emulsions (5.0 to 5.4) agrees with previous data obtained in the presence of either short, medium, or long chain triglycerides.[10,11,22] It is noteworthy that the enzyme activity might be higher in the case of small emulsified droplets at very low pH (pH 3.0 or below), as found in the stomach just after food intake.

The purified gastric lipase was shown to have a lower affinity for the pure MCT emulsion than for the pure LCT emulsion (apparent K_m (m^2/L) ratio : 10.3). This means that the differences observed are basically due to the nature of the substrate, or in other words, to the interface properties. The affinities of gastric lipase were comparable for the two mixed MCT/LCT emulsions with different mean droplet sizes (3.18 versus 0.46 μm) and were close to that obtained for the pure LCT emulsion *(Table 2)*. In a previous study,[14] the pancreatic lipase affinity was comparable for the four emulsions studied in the conditions prevailing in the duodenum. From physicochemical data,[30] we can assume that the concentration of trioctanoin in the surface monolayer of the particles must be several-fold higher than that of a long chain triglyceride. Therefore, it appears that the low interfacial concentration of LCT might be enough to give comparable enzyme affinities either for pure LCT or mixed MCT/LCT emulsions. Moreover, the binding measurements reported herein *(Table 3)* demonstrate that the extent of binding of gastric lipase onto the coarse or the fine mixed MCT/LCT emulsion was similar. Thus, taken together the present observations indicate that the chemical composition of the surface monolayer more markedly influences the gastric lipase affinity than other interface properties, such as droplet size or surface pressure, in the range of droplet sizes used.

The maximal velocity (V_{max}) of the purified rabbit gastric lipase recorded on the pure MCT emulsion was three-fold higher than that determined on the pure LCT emulsion. A comparable difference was observed with the human enzyme provided in the form of gastric juice (data not shown). A higher rate of hydrolysis of trioctanoin versus triolein by rat lingual lipase[12] or medium chain versus long chain triglycerides by rabbit gastric lipase[22] has been reported. This is also true for tributyrin versus long chain triglyceride hydrolysis by human and rabbit gastric lipases[10,11,22] or porcine pancreatic lipase.[14] Similar data have been obtained in the case of lipoprotein lipases and hepatic lipases with MCT and LCT emulsions.[30] Thus, in general, various lipases hydrolyze short chain and medium chain triglycerides more readily than long chain triglycerides.

We observed that the V_{max} exhibited by gastric lipase was slightly but significantly higher on the fine MCT/LCT emulsion than on the coarse MCT/LCT emulsion *(Figure 2, Table 2)*. Thus, in the range studied, particle size alters the catalytic step of the enzyme reaction as previously shown with pancreatic lipase,[14] but to a lower extent. Nevertheless, other studies have shown that the activity of human gastric lipase on diglyceride monolayers at the tributyrin/water interface[11] markedly changed when the interfacial tension varied. The V_{max} determined in our study with the two mixed MCT/LCT emulsions showed intermediate values between those of the pure LCT and MCT emulsions. This was previously observed with pancreatic lipase.[14] Possibly the presence of even low amounts of long chain triglycerides in the surface monolayer is enough to reduce the mobility of MCT in phospholipids (and sugar-esters), as has been recently suggested,[30] which markedly decreases the rate of hydrolysis of MCT by gastric lipase.

The observed inhibition of gastric lipase activity as a function of time *(Figure 3)* was likely due to the progressive release of protonated fatty acids, as suggested by other studies.[10,12,13] The concentration of bovine serum albumin used (30 μmol/L) was enough to prevent enzyme denaturation at the droplet interface[15] but appears definitively too low to bind appreciable amounts of generated free fatty acids (up to 5 mmol/L after 30 min reaction) *(Figure 3)*, and consequently, to prevent enzyme inhibition. The rate of inhibition of gastric lipase was more marked with the coarse than with the fine mixed MCT/LCT emulsion, suggesting that this process might be surface area-dependent. When externally adding free fatty acids (*Figure 4*), a marked inhibition of gastric lipase was observed. The inhibition was more drastic with caprylic acid than with oleic acid and with the coarse mixed emulsion. For instance, the bulk concentration of caprylic acid that induced 50% inhibition of gastric lipase activity was 1.6 and 5.2 mmol/L, respectively, with the coarse and the fine mixed emulsion. When calculated regarding emulsion surface areas, the figures given for the two emulsions (10.1 to 19.2 μmol free fatty acids/m^2) were in the same range, suggesting the key role of the surface concentration of free fatty acids in the inhibition process. This concept is supported by the observation that the free fatty acids present in a phospholipid-stabilized triglyceride emulsion more preferentially accumulate in the surface monolayer rather than in the hydrophobic triglyceride core, even in a protonated form.[31] Moreover, the transfer of free fatty acids from the emulsion surface to the phospholipid vesicles or albumin in the aqueous phase was shown to be markedly reduced at acidic pH,[31] as in the human stomach. The less markedly inhibitory effect of oleic acid, as compared with caprylic acid, may be understood by considering its likely lower surface to core partitioning in the droplets and thus its more reduced surface accumulation in the particles.

After passing through the pylorus, the pre-hydrolyzed fat emulsion reaches the duodenum, where the bile and pancreatic secretions complete the hydrolysis of triglycerides under normal physiological conditions.[4] It was pre-

viously demonstrated in vitro that pancreatic lipase hardly hydrolyzes complex phospholipid-stabilized emulsions[15,19] or milk fat globules[13,16] under unfavorable conditions such as reduced calcium ion or bile salt concentrations or suboptimal colipase supply. Such conditions may occur in several pathological situations in human subjects. The lag time measured in vitro represents the time needed by the enzyme to bind onto the particles and to reach the maximal rate of triglyceride hydrolysis. We have confirmed our previous observation[14] that the lag time of pancreatic lipase is greater with the fine compared with the coarse mixed MCT/LCT emulsion. Moreover, we have shown that such a long lag time is due to a lack of binding of pancreatic lipase onto the emulsified droplets, in agreement with other data.[19] The increased lag time can be overcome when gastric lipase has pre-hydrolyzed the emulsions sufficiently to generate about 10 to 15 μmol free fatty acids/m^2 of available emulsion surface area. Other studies had pointed out that free fatty acids might reduce or suppress the lag time.[13,15,19] The effects of generated fatty acids on the maximal velocity of pancreatic lipase still remains very controversial. Our present findings indicate that in the case of complex mixed MCT/LCT emulsions, a medium chain fatty acid (caprylic acid) slightly increases pancreatic lipase activity and a long chain fatty acid (oleic acid) slightly reduces the enzyme activity on the fine emulsion, whereas it markedly depresses the enzyme activity on the coarse emulsion. Once more, this effect appears to be surface-area dependent. We did not observe any marked activating effect of free fatty acids on pancreatic lipase activity as previously suggested.[15]

Some practical consequences of nutritional and clinical relevance can be drawn from this in vitro study and another recently dedicated to pancreatic lipase.[14] A mixed MCT/LCT emulsion was found to be a suitable substrate for gastric or pancreatic lipases. The size of the emulsified droplets could be a key parameter under gastric conditions, especially at very acidic pH as well as in the duodenum. The inhibition of gastric lipase activity induced by generated free fatty acids being surface area-dependent appears to favor the fine emulsion over the coarse as a better substrate. Moreover, it seems reasonable to recommend that addition of free fatty acids as emulsifiers into emulsions should be avoided, especially those that are tube-fed into the stomach of subjects. There gastric lipase is the predominant lipolytic enzyme, as in pancreatic insufficiency. The presence of MCT reduces the extent of phospholipid removal from the emulsified droplets in the presence of bile salts, which occurs in the duodenum. Pre-hydrolyzing the emulsions under acidic conditions by gastric lipase slightly increases the activity of pancreatic lipase and facilitates the enzyme binding onto the emulsified droplets, as do calcium ions.[14] Thus, calcium should be added in a surface-dependent manner to improve the binding and activity of pancreatic lipase on the emulsified droplets.

In conclusion, the physico-chemical properties of fat emulsions markedly influence triglyceride hydrolysis catalyzed by gastric and pancreatic lipases.

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