Effects of droplet size, triacylglycerol composition, and calcium on the hydrolysis of complex emulsions by pancreatic lipase: an in vitro study

Martine Armand, Patrick Borel, Pascale Ythier, Guy Dutot, Christian Melin, Michèle Senft, Huguette Lafont, and Denis Lairon

Unité de Recherches sur le Transport des Lipides, INSERM (National Institute of Health and Medical Research), Marseille and Laboratoire de Recherche-Developpement, Clintec-Cernep, Le Pessis Robinsson, France

The pancreatic lipase-catalyzed hydrolysis of complex lipid emulsions dedicated to enteral nutrition in humans was studied in vitro. The four complex emulsions used had variable triglyceride composition and particle sizes, and a similar phospholipid content. They contained triglycerides in the form of pure medium-chain triglycerides (MCT), pure long-chain triglycerides (LCT), or a mixture of MCT and LCT (MCT/LCT 20/80 wt/wt). The mean droplet sizes of the emulsions were 0.19 μm (MCT), 0.43 μm (LCT), 0.46 μm, or 3.18 μm (MCT/LCT). The phase distribution of phospholipids (emulsified particles/aqueous medium) was shown to be related to the saturation of the available triglyceride interface area. The presence of MCTs appears to protect phospholipids from being removed from the droplet surface by bile salt micelles. The affinity of pancreatic lipase for the emulsions was comparable, and calcium ions were shown to play a key role in suppressing the lag phase in a surface area-dependent manner. The enzyme velocity was inversely related to the mean particle size of the emulsions. Pure MCTs were hydrolyzed faster than pure LCTs. With comparable sizes, a mixed MCT/LCT emulsion was hydrolyzed more slowly than a pure MCT emulsion. In conclusion, varying the mean droplet size or the triglyceride composition of emulsions affects their hydrolysis rate catalyzed by pancreatic lipase. The present findings could help in preparing new emulsions for enteral feeding, especially for patients with a reduced digestive capacity.

Keywords: *emulsion; triglyceride; hydrolysis; pancreatic lipase; droplet size; in vitro*

Introduction

There is an increasing use of artificial emulsions for human enteral nutrition because tube-feeding maintains an optimal activity of the gastrointestinal tract as compared with parenteral nutrition. A wide spectrum of enteral formulas are already available, but emphasis is now placed on disease-specific formulas.[1] In some patients, key steps involved in fat digestion and absorption[2] are damaged, thus leading to fat malabsorption, reduced energy supply, steatorrhea, and severe health complications [1,3]. Such patients include those with extensive stomach and small-bowel resection, severe exocrine pancreas insufficiency (especially chronic pancreatitis and cystic fibrosis), hepatic dysfunctions and reduced bile secretion, or severe abnormalities of intestinal mucosa (untreated coeliac disease).

In the case of parenteral nutrition, the emulsions must have small particles, mainly because of embolic risk. This constraint does not occur in the case of enteral nutrition and, thus, emulsions can be made of with much larger particles. Nevertheless, the particle size of the emulsions may be an important parameter because the activity of the lipolytic enzymes is highly dependent on the properties of the substrate-water interface.[4] Thus, the characteristic feature of gastroin-

Address reprint requests to Dr. Denis Lairon at the Unité 130 de l'INSERM, Centre INSERM-Viton 18 Avenue Mozart, 13009 Marseille, France.
Received June 4, 1991; accepted December 9, 1991.

testinal lipases from either stomach or pancreas origin is their specificity to act on insoluble emulsified substrates.[4–6] Consequently, the specific interface area (m^2/g) of the emulsion, which increases when the particle size decreases, governs the activity of lipases on emulsified triglycerides more than the substrate concentration does.[7] We do not know what the extent is of fat emulsification when dietary lipids come into the duodenum and thus, very little is known about the optimal size of emulsions for enteral nutrition. We can assume that, in normal physiological conditions, the mechanical forces and the partial triglyceride hydrolysis elicited by preduodenal lipases[6] in the stomach generate an emulsion with a droplet size spectrum ensuring an efficient activity of pancreatic lipase. In other circumstances, when tube-feeding is provided directly into the duodenum, it becomes very important to infuse emulsions with suitable particle size and composition to promote an optimum activity of pancreatic lipase and consequently, an efficient fat assimilation.

After demonstrating that bile phospholipids play an important role in fat lipolysis both in vivo[8] and in vitro,[9,10] we established that a condensed monolayer including phospholipids and cholesterol is built on the particle hydrophobic surface in the conditions prevailing in the upper small-intestine.[11–13] Recent data on the lipid composition of human duodenal aspirates after a test meal reinforce this view.[14] In that context, phospholipid-stabilized triglyceride emulsions were subjected to in vitro hydrolysis by pancreatic lipase in various conditions.[13,15–18] Because of their amphipatic properties, phospholipids are only located in either the surface monolayer of the emulsion particles[19] or in the aqueous medium. To increase the long-term stability of emulsions, the usual industrial processes use an excess amount of phospholipids[15,18,20,21] without consideration of the possible consequences on lipolytic enzymes, as suggested by other studies.[11–13,15,16,18,21] This very important point was thus reexamined in detail by using emulsions with variable droplet sizes.

In fact, since the pioneer works of Benzonana and Desnuelle,[7,22] the activity of pancreatic lipase has been measured on various triglyceride substrates without consideration of the emulsion droplet size. However, physico-chemical studies do indicate that the interface properties are key factors governing lipase binding and activity.[11,23–26] Therefore, we studied the effect of the size of the substrate emulsified droplets (from 0.19–3.18 μm), made of mixtures with comparable or different lipid compositions, on pancreatic lipase affinity and activity.

Although no ion seems to be required as the absolute lipase cofactor, calcium ions have been implicated in the mechanism of action of pancreatic lipase for a long time.[13,15,18,22,27–30] Calcium ions can either modulate lipase velocity or the lag phase until the maximal enzyme activity is reached. Calcium may decrease electrostatic repulsions occurring at the emulsion-water interface, but excess calcium can induce coalescence of emulsified particles. In fact, the role of calcium ions is still unclear and thus, the calcium dependence of the lipolytic reaction was determined in the presence of different emulsified substrates.

Nowadays, complex emulsions made of mixtures of medium-chain triglycerides (MCTs) and long-chain triglycerides (LCT) are used more often because MCT assimilation is less dependent on digestive tract disorders and LCTs are required as a source of essential fatty acids. Measurements of pancreatic lipase activity have already been performed either on pure short-chain[13,31,32] or MCT,[33] as well as on pure LCT.[5,7,13,22,27] The lipolysis of mixed MCT/LCT emulsions was only very recently studied with lipoprotein lipase and hepatic lipase.[21,34] Because no data were available about the activity of pancreatic lipase on mixed MCT/LCT emulsions, we determined in the present study the effect of the nature of the substrate, i.e., pure or mixed MCTs and LCTs, in the form of stable and calibrated emulsions.

The results obtained here indicate that the emulsion droplet size influences the phospholipid phase partition and governs the calcium dependence of the lipolytic reaction as a function of the surface area, and that pancreatic lipase velocity increases when the mean droplet size decreases.

Materials and methods

Emulsions

The four emulsions used were made of pure MCT (0.19 μm mean droplet size), pure LCT (0.43 μm mean droplet size) or mixed MCTs/LCTs (0.46 μm or 3.18 μm mean droplet sizes). The emulsions used came from Clintec-Cernep Laboratories (Le Plessis Robinsson, France) and were prepared using a high pressure homogenizer (Ets Manton, Gaulin, France). The chemical compositions and some physico-chemical parameters of the emulsions are given in *Table 1*. These emulsions were complex mixtures made to meet nutritional requirements more accurately. Triglycerides were either MCT containing 48.5% C8 : 0 and 39.7% C10 : 0, or LCT essentially provided by various vegetable oils. The fatty acid composition of the LCT mixture was 11.5% C16, 82.6% C18, 2.9% C20, and 2.5% C22, with 54% monounsaturated and 23% polyunsaturated fatty acids. The mixed MCT/LCT emulsions contained (wt/wt) 20% MCT and 80% LCT or (mol/mol) about 34% MCT and 64% LCT. Soybean lecithins and sugar esters were used as emulsifiers *(Table 1)*. Ivelip (0.44 μm mean droplet size), a commercial emulsion suitable for parenteral nutrition (Cernep-Synthelabo) was used as reference in only one experiment. It basically contained soybean oil (20%), egg lecithin (1.2%), sodium oleate (0.03%), and glycerol (2.5%).

The distribution of the emulsion droplet sizes was determined by using a particle-size analyzer (Capa 700, Horiba, Kyoto, Japan). Emulsions were diluted twice in distilled water to obtain an optical density between 0.9 and 1.0 at 560 nm. Measurements were done using gradient mode analysis at a constant centrifuge acceleration rate (120 rpm/min) to allow an accurate measurement of large particles (10 μm and above) as well as small particles (0.05 μm). Results are given in the form of a frequency distribution graph *(Figure 1)*. Pure MCT, LCT, and fine MCT/LCT emulsions showed a narrow distribution, expressed in fractions of total volume or area. The mean particle size of the pure MCT emulsion was slightly lower (0.19 μm) than that of the pure

Table 1 Compositions and mean droplet sizes of emulsions

Component (weight %)	Emulsions			
	Coarse MCT/LCT	Fine MCT/LCT	MCT	LCT
Medium-chain triglycerides	4.00	4.00	20.00	0.00
Long-chain triglycerides (mixed oils)	16.00	16.00	0.00	20.00
Emulsifiers (soybean lecithins, sugar esters)	4.23	4.23	4.23	4.23
Mean droplet size (μm)[a]	3.18	0.46	0.19	0.43

[a]Mean droplet sizes were calculated by the particle-sizer software (Capa 700, Horiba) from the droplet size distribution expressed as a function of total droplet volume.

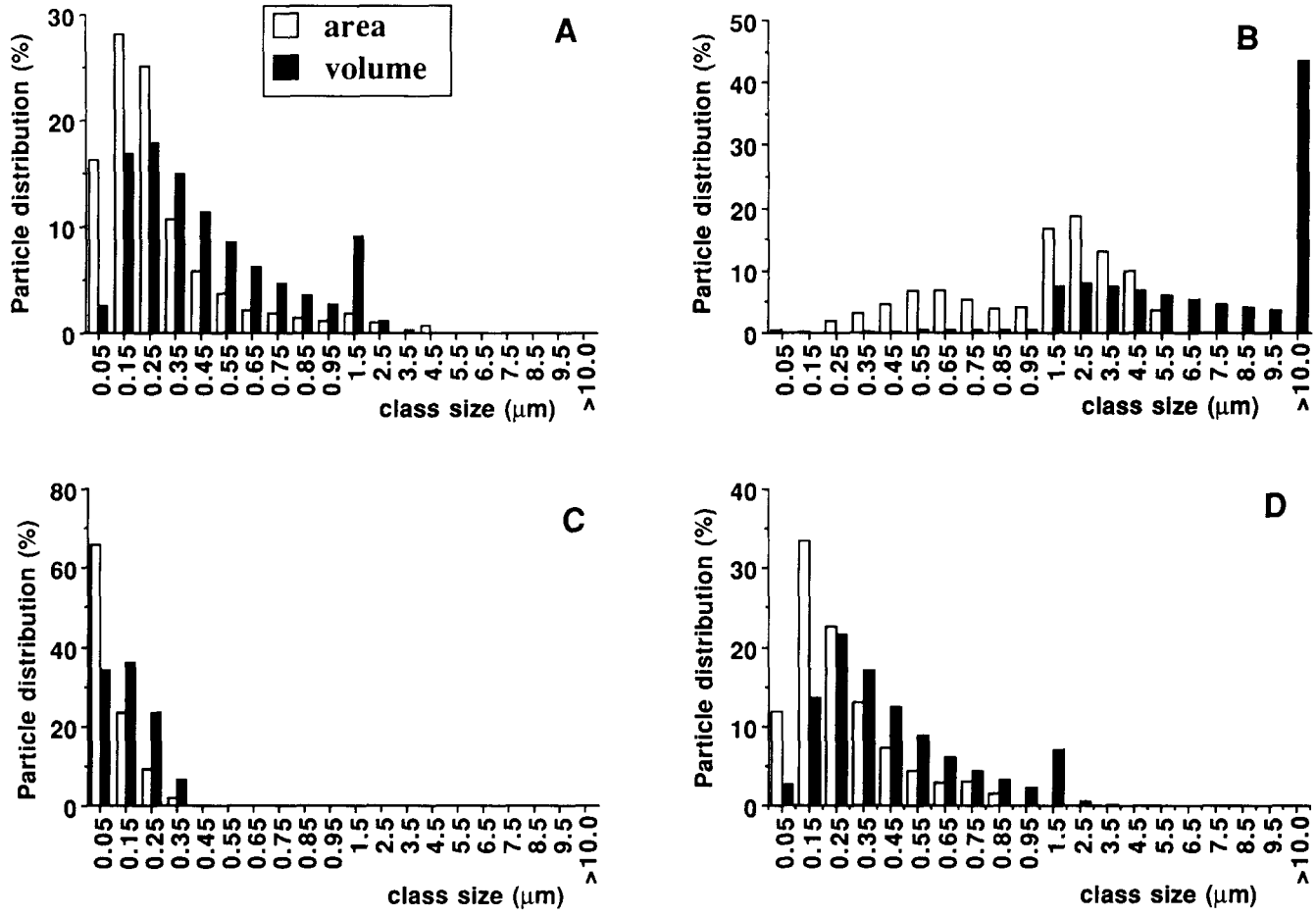

Figure 1 Droplet size distribution of emulsions measured by using a particle size analyser (Capa 700, Horiba). Results are expressed as percentage of total volume or area. A, Fine MCT/LCT emulsion. B, coarse MCT/LCT emulsion. C, pure MCT emulsion. D, pure LCT emulsion. Experimental conditions are described in Materials and methods. Calculated mean diameters are given in *Table 1*.

LCT (0.43 μm) or the fine MCT/LCT emulsion (0.46 μm) because of differences in the physico-chemical properties of the emulsion made with MCT alone. The coarse MCT/LCT emulsion showed a wider spectrum because the high pressure homogenizer failed to produce coarse emulsions with a narrow spectrum. Nevertheless, more than half of the area was represented by droplets ranging from 1.5 μm to 4.5 μm.

Lipid partitioning and analysis

The phase partition of phospholipids present in the emulsions, i.e., the distribution of phospholipids between the emulsified droplets (cream) and the aqueous phase (infranatant) was determined.[15,20,21] One mL of each emulsion was mixed with 14 mL distilled water or with the pancreatic lipase reaction buffer (pH 7.50) that contained 6 mmol/L Na taurodeoxycholate, 10 mmol/L $CaCl_2$, 150 mmol/L NaCl, and 2 mmol/L Tris-HCl. The mixture was mechanically stirred for 5 min; 7 mL of the mixture were put in a polyallomer centrifuge tube and overlayered with 1 mL of distilled water. Samples were centrifuged (from 28,000–160,000*g* and for 4 min–22 hr) in a SW 40 Ti rotor at 22° C in a Beckman L 2-65B ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA). The ultracentrifugation conditions were adapted to

each individual emulsion in the presence of water or the bile salt buffer (pH 7.50) to prevent emulsions from breaking (absence of an oil phase overlayering the cream). The centrifuge was operated without braking. Under these conditions, about 97%–99% of the triglycerides were found in the emulsion phase and consequently there was no important contamination of the infranatant aqueous phase with triglyceride molecules (data not shown).

Lipids were extracted using the method of Folch et al.[35] Phosphorus was measured after mineralization[36] and triglycerides were assayed using an enzymatic method[37] in the form of PAP 150 assay kit (BioMerieux. Marcy-l'Etoile, France).

Pancreatic lipase activity measurements

Porcine pancreatic lipase (E.C.3.1.1.3) was purchased from Boehringer Mannheim (Mannheim, Germany). It was 95% pure as checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and its specific activity was 1370 units/mg of protein. Porcine pancreatic colipase came from Boehringer Mannheim. The specific activity of this pure preparation was 1590 units/mg of protein. In both cases, one unit was defined as 1 μ equivalent fatty acid titrated per min.

Porcine pancreatic lipase activity was measured potentiometrically with a pH-stat titrator (Metrohm, Herisau, Switzerland) by titration of released fatty acids with 0.02 mol/L NaOH at pH 7.50 at 37° C. The molar ratio between lipase (20 nmol/L) and colipase (100 nmol/L) was kept constant (1:5), except in one experiment. The reaction volume was 15 mL containing 150 mmol/L NaCl, 10 mmol/L $CaCl_2$, 6 mmol/L Na taurodeoxycholate, 2 mmol/L Tris-HCl (pH 7.50), and 200 μL of the studied emulsion (2.67 g/L triglycerides), unless otherwise indicated. The initial velocity of pancreatic lipase was measured. In conditions in which measurable lag times occurred, lipase activity was recorded until the maximum enzyme velocity was reached. The time needed to obtain a linear recording was defined as the lag time.[15] The standard reaction conditions (taurodeoxycholate and $CaCl_2$ concentrations and lipase:colipase molar ratio) provided minimum lag time[15] and reproducible measurements. Because titration of fatty acid depends on the pH, the nature of the fatty acid, and various other parameters,[22] it was necessary to correct the apparent activity recorded at pH 7.50 by estimating the percentage of fatty acids that were actually titrated at this pH value, as shown earlier.[16] Thus, virtually all the fatty acids released under lipolysis were titrated at the end of the reaction[38] at pH 10.50, in the particular conditions of each type of experiment. True enzyme activity values were calculated. All measurements of lipase activity and lag time were done in duplicate.

To determine the pH dependence of pancreatic lipase activity, the pH of the assay medium was adjusted from pH 5.00 to pH 10.00 with NaOH (1 mol/L) before measuring pancreatic lipase activity as previously described. To measure the effect of increasing enzyme concentrations, various amounts of pancreatic lipase (from 10–160 nmol/L) were added to the assay mixture, with a constant 1:5 lipase:colipase ratio. To determine the effect of calcium concentration on the lag time and on pancreatic lipase velocity, various concentrations of $CaCl_2$ (from 0–20 mmol/L) were used and the lipase:colipase molar ratio was only 1:2 (lipase 20 nmol/L, colipase 40 nmol/L) because excess colipase was shown to markedly reduce lag time.[15] Measurements of pancreatic lipase velocity were performed in the presence of increasing emulsion concentrations under conditions suppressing lag time, as described above. Maximum enzyme velocity (Vmax) and apparent Km values were calculated using Lineweaver-Burk plots.

Results

Stability of emulsions in the presence of bile salts

As shown in *Figure 2*, the particle size distribution of three emulsions was determined after a 15 min incubation in the bile salt buffer. As compared with incubations in distilled water, no marked change in the particle size distribution was observed either with the coarse MCT/LCT emulsion *(Figure 2A)*, the pure LCT emulsion *(Figure 2B)*, or the pure MCT emulsion *(Figure 2C)*.

Phase partitioning of phospholipids

After incubation in distilled water *(Figure 3)*, the proportion of phospholipids in the infranatant was inversely related to the mean size of the emulsion

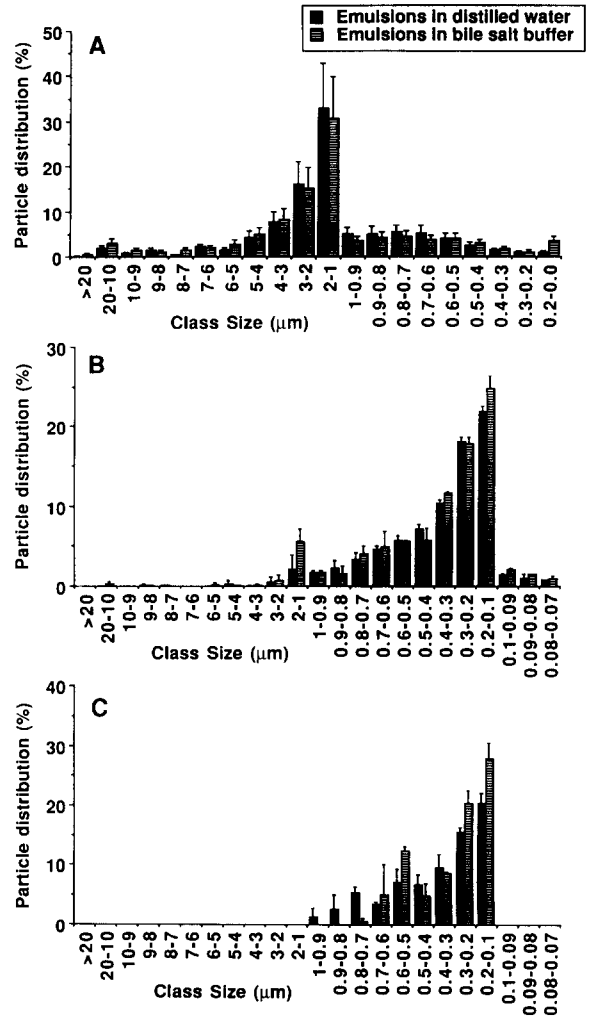

Figure 2 Droplet size distribution of emulsions after 15 minutes incubation in distilled water or in the presence of buffer containing 2 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L $CaCl_2$, 6 mmol/L taurodeoxycholate, pH 7.50. **A**, Coarse MCT/LCT emulsion. **B**, pure LCT emulsion. **C**, pure MCT emulsion. Each value represents the mean ± SE of two determinations.

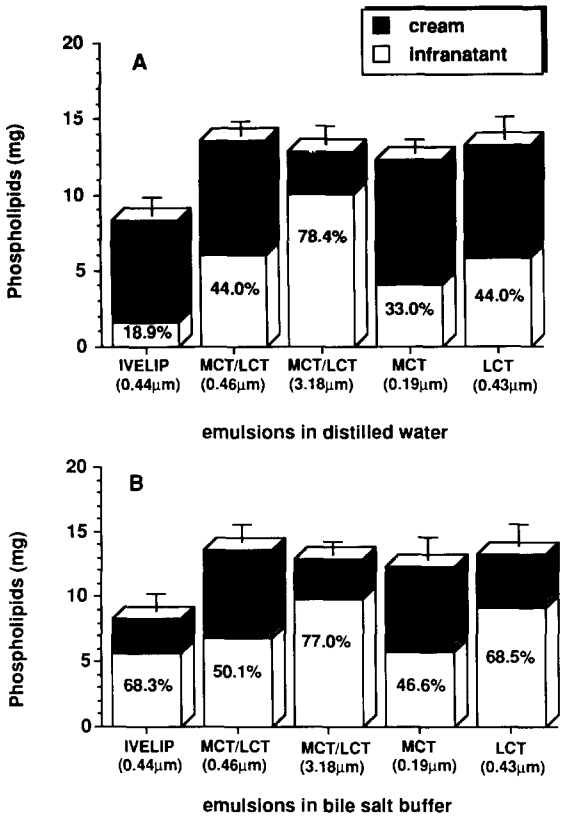

Figure 3 Partitioning of phospholipids between the emulsion phase (cream) and the soluble phase (infranatant). A, phospholipid distribution after incubation in distilled water. B, phospholipid distribution after incubation in pancreatic lipase assay medium (2 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L $CaCl_2$, 6 mmol/L taurodeoxycholate, pH 7.50). For more details, see Materials and methods. Each value represents the mean ± SE of two determinations.

droplets: 33.0%, 44.0%, 44.0%, and 78.4% of the phospholipids were present in the infranatant with the smallest emulsion (MCT, 0.19 μm), the medium emulsions (LCT, 0.43 μm and MCT/LCT, 0.46 μm), and the largest emulsion (MCT/LCT, 3.18 μm), respectively. As compared with other emulsions with comparable droplet size, the proportion of phospholipids in the infranatant for the Ivelip emulsion used as control was two times lower. In fact, Ivelip contained only one emulsifier (egg lecithins, 1.2%) as compared to the mixture of soybean lecithins and sugar esters in the other emulsions used.

Incubating emulsions in the pancreatic lipase reaction buffer containing bile salt *(Figure 3B)*, as compared with incubating them in distilled water, induced a marked increase in the phospholipid content of the infranatants with the pure MCT emulsion, the pure LCT emulsion, and Ivelip. No marked changes were observed in the case of the fine and the coarse mixed MCT/LCT emulsion.

pH dependence of porcine pancreatic lipase activity

The enzyme activity curves obtained either on the fine MCT/LCT or on the coarse MCT/LCT emulsions were not markedly different, as shown in *Figure 4*. The maximum activities were reached at pH 7.00–7.50 for both mixed emulsions. At pH 5.00, pancreatic lipase kept 35.0% and 25.0% of its maximal activity with the coarse or the fine MCT/LCT emulsion as substrate, respectively. A marked difference was observed at pH 9.0: pancreatic lipase kept 56.0% of its maximum activity on the fine MCT/LCT emulsion but exhibited only 4.0% of its maximum activity in the presence of the coarse MCT/LCT emulsion.

Calcium dependence of pancreatic lipase activity

The activity of pancreatic lipase either on the fine or the coarse MCT/LCT emulsion increased 2.3- or 3.7-fold, respectively, when the calcium concentration increased from 0 to 2 mmol/L *(Figure 5A)*. When the calcium concentration further increased to 10 mmol/L, the lipase velocity remained constant when the substrate was the coarse MCT/LCT emulsion but increased steadily with the fine MCT/LCT emulsion until a maximum value was reached (5.7-fold increase). Further increasing the calcium concentration did not change the lipase activity.

The lag time, i.e., the time needed to reach the steady state maximum velocity, was measured. As shown in *Figure 5B*, the lag time of the reaction, measured in the presence of a reduced excess of colipase (lipase:colipase ratio: 1:2) and without added $CaCl_2$ in the reaction medium, was much higher with the fine MCT/LCT emulsion (17.4 min) than with the coarse MCT/LCT emulsion (5.1 min). The lag time for both emulsions dramatically decreased as the $CaCl_2$ con-

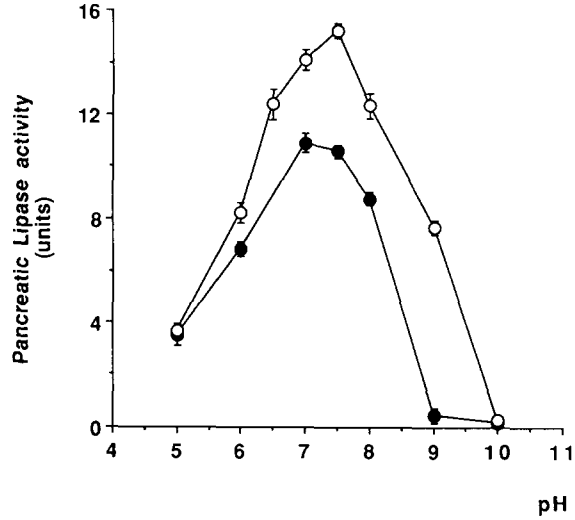

Figure 4 Effects of pH on the hydrolysis rate of fine MCT/LCT (○) and coarse MCT/LCT (●) emulsions by pancreatic lipase. The porcine pancreatic lipase (20 nmol/L) and colipase (100 nmol/L) (molar ratio 1:5) were incubated at 37° C in a medium containing 200 μL emulsion and reaction buffer containing 2 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L $CaCl_2$, and 6 mmol/L taurodeoxycholate (final volume:15 mL) at the indicated pH. Lipase activity was determined as indicated in Materials and methods. Each point represents the mean ± SE of two determinations.

centration increased and became negligible above 2 mmol/L $CaCl_2$.

Kinetics

When we increased the pancreatic lipase concentration in the reaction medium (from 10 to 160 nmol/L) at a fixed 1:5 lipase:colipase ratio, the lipase activity increased linearly when the substrate was the fine or the coarse MCT/LCT emulsion (data not shown).

Measurements of pancreatic lipase activity in the presence of increasing amounts of the four different emulsions are shown in *Figure 6*. Although lipase-catalyzed reactions occur in a heterogeneous system, Michaelis-Menten curves were obtained when the substrate concentration was expressed in triglyceride mass concentration (g/L). Pancreatic lipase activity was higher on the fine mixed MCT/LCT emulsion than on the coarse mixed MCT/LCT emulsion *(Figure 6A)*. The Vmax values derived from Lineweaver-Burk plots are given in *Table 2*. The highest lipase Vmax was obtained in the presence of the pure MCT emulsion and was markedly higher than that measured on the pure LCT emulsion *(Figure 6B* and *Table 2)*.

From the calculated apparent Kms *(Table 2)* derived from linear Lineweaver-Burk plots of the enzyme velocity as a function of the substrate concentration expressed in interfacial area (m^2/L), it appeared that the affinity of pancreatic lipase for the fine MCT/LCT

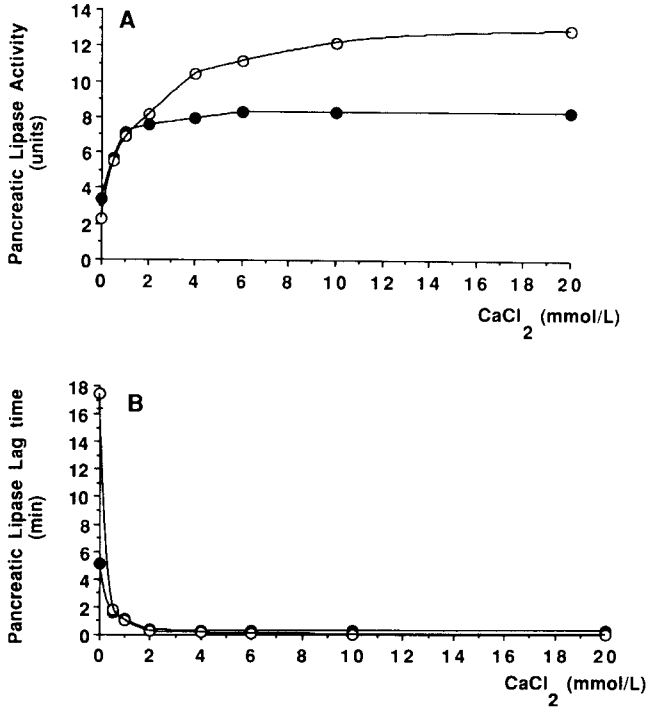

Figure 5 Effect of calcium concentration on **(A)** pancreatic lipase activity (lipase:colipase molar ratio 1:2) and on **(B)** pancreatic lipase lag time (see Materials and methods) wtih the fine MCT/LCT emulsion (○) and the coarse MCT/LCT emulsion (●). Reactions were carried out as indicated in the legend for *Figure 4*, with calcium concentrations ranging from 0–20 mmol/L. Each point represents the mean ± SE of two determinations.

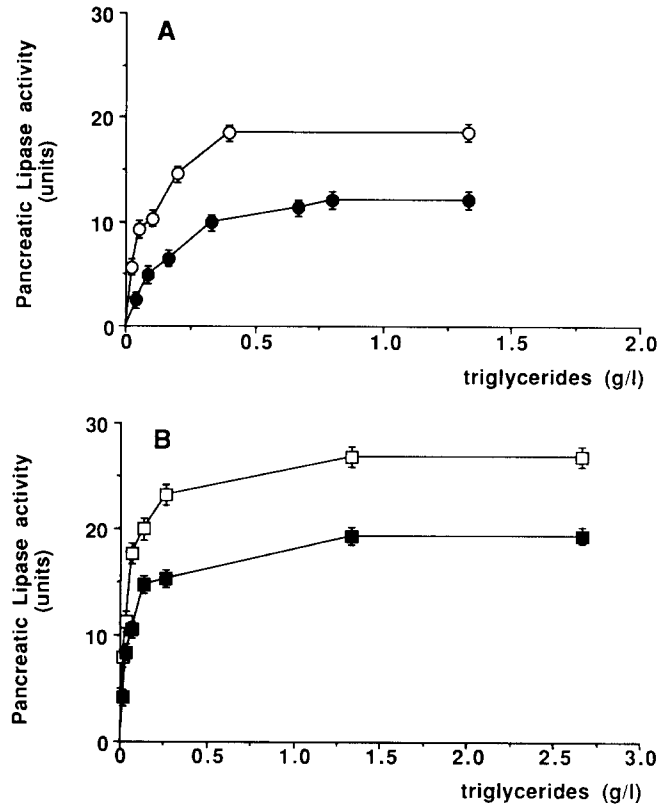

Figure 6 Effect of triglyceride concentration on the lipolytic activity of pancreatic lipase. The incubation mixture consisted of the indicated amount of substrate, pancreatic lipase (20 nmol/L), and pancreatic colipase (100 nmol/L). Assay conditions were as indicated in the legend for *Figure 4*. **(A)** fine MCT/LCT emulsion (○) and coarse MCT/LCT emulsion (●). **(B)** pure MCT emulsion (□) and pure LCT emulsion (■). Each point represents the mean ± SE of two determinations.

Table 2 Pancreatic lipase kinetic parameters

Emulsions	Vmax (units)	Apparent Km (m^2/L)
Coarse MCT/LCT (3.18 μm)	14.6	0.59
Fine MCT/LCT (0.46 μm)	19.2	1.17
MCT (0.19 μm)	27.0	2.03
LCT (0.43 μm)	21.3	1.47

emulsion (0.46 μm) was comparable to that determined for the coarse MCT/LCT emulsion (3.18 μm). The apparent Km calculated with the pure MCT emulsion was close to that determined with the pure LCT emulsion *(Table 2)*.

Discussion

Behavior of complex emulsions in the presence of bile salts

To obtain very stable emulsions, excess phospholipids and other surfactants such as sugar esters are added to industrially prepared emulsions. This leads to a two-phase system with the coexistence of triglyceride drop-

lets surrounded by a surface-mixed monolayer made mainly of phospholipids[19] and dispersed low molecular-weight phospholipid vesicular structures. The latter are in equilibrium with the phospholipids present on the droplet surface. We do not know the exact behavior of sugar esters in such a two-phase system. We observed that the amount of excess phospholipids in the aqueous phase was inversely related to the available interface provided by the different emulsions studied. As it certainly occurs in the duodenum,[2] the two-phase system evolved when bile salts in micellar solution (6 mmol/L) were added to the aqueous phase. Due to the high affinity of bile salts for phospholipids, phospholipid vesicles were likely dispersed at various extents in the form of mixed micelles,[14] and some bile salt molecules bound to the emulsion surface, expelled a part of the phospholipid molecules from the surface of the emulsion, and thus intercalated in the surface monolayer, as shown earlier.[11,12] This process did not result in noticeable changes in the emulsified droplet size even with the least stable emulsion used (coarse mixed MCT/LCT emulsion) nor with the pure LCT and MCT emulsions in which phospholipids are partially removed in the presence of bile salts. As the emulsions used also contain sugar esters, we should not conclude that this observation is valid for every kind of dietary fat mixture. In agreement with previous data obtained with Intralipid (Kabi-vitrum, Paris) as the emulsified substrate,[15,18,20] we observed here with Ivelip (soybean triglycerides) and the pure LCT emulsion a comparable drastic increase in the phospholipid concentration in the aqueous phase in the presence of bile salt. It is noteworthy that the amplitude of this phenomenon was lower in the case of the pure MCT emulsion and almost nonexistent in the case of both mixed MCT/LCT emulsions. Thus, these observations point out that the mean droplet size of emulsions (range 0.19–3.18 μm) containing sugar esters hardly influences the depletion of phospholipids from the interface promoted by bile salt micelles, but that emulsion composition plays a key role in this process. In fact, the addition of 20% (wt/wt) MCT to LCT, giving mixed MCT/LCT emulsions with a 1:2 molar ratio, stabilizes the emulsion interfacial monolayer by markedly decreasing the phospholipid removal. This may be explained by the basic observation[34] that MCTs preferentially accumulate in the surface-mixed monolayer of emulsions rather than LCTs (11 versus 3 mol %). Moreover, the stabilizing effect of MCTs seems explainable by the fact that MCTs have collapse pressure in monolayers of about 20–35 mN/m; these values are close to those of lecithins (35–50 mN/m), but are much higher than those of LCTs (10–15 mN/m).[12,24] This observation might be taken into account when preparing emulsions dedicated to tube-feeding.

Kinetics of pancreatic lipase acting on complex emulsions

Some key factors involved in triglyceride hydrolysis of various emulsions by pancreatic lipase were studied. The optimum pH of the enzyme was not affected by the emulsion droplet size of mixed MCT/LCT emulsions.

The affinities of pancreatic lipase for the four emulsions studied were in the same order of magnitude *(Table 2)* when the substrate concentration was expressed in interface area concentration (m^2/L), as done earlier.[7] These data point out that the emulsion droplet size, at least in the range studied (0.19 to 3.18 μm), does not alter the affinity of the enzyme for the emulsion interface. Moreover, the triglyceride composition of the droplets, either MCT or LCT, does not significantly alter the affinity. This confirms previous determinations performed in the presence of bile salt micelles and colipase, showing comparable Km values obtained with either short-chain (tributyrin) or long-chain triglycerides.[32] This suggests that in the conditions prevailing in the small intestine and for given emulsifiers, differences in the triglyceride composition of the monolayer surrounding the triglyceride core of the droplets might not markedly influence the colipase-lipase-emulsion interactions.

The measurements of apparent Vmax provided relevant information. The effect of the droplet size was directly observed by comparing the Vm values obtained with the fine and coarse emulsions made with the same MCT/LCT mixture *(Table 2)*. Moreover, when comparing the pancreatic lipase Vmax obtained with the four emulsions used, it appeared that Vmax was related to the droplet size of the emulsions as illustrated in *Figure 7*. Vmax decreased when the mean droplet size of the emulsion increased (Vmax = −9.026 log diameter + 18.301, R = 0.88). This was particularly obvious at sizes under 0.6 μm. From the data obtained from monolayer studies we can assume that the packing density of the (sugar esters)-phospholipid-triglyceride monolayer surrounding the triglyceride droplets determines a high surface pressure in such phospholipid-stabilized emulsions.[24] Values of 33 mN/m were reported,[25] with up to 38 mN/m in the presence of bile phospholipids and bile salts.[13] However, when high lipid packing and surface pressure

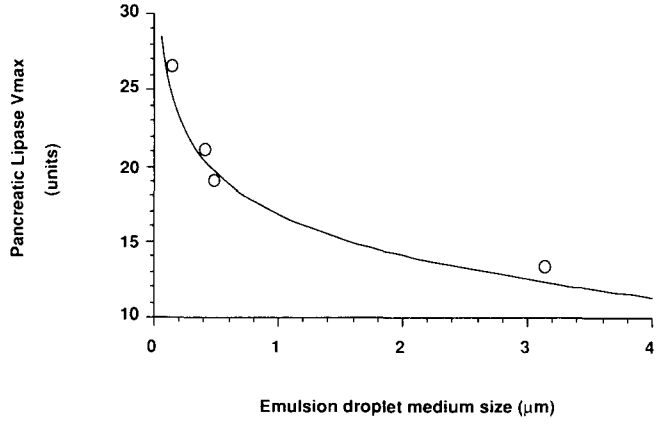

Figure 7 Relationship between emulsion mean droplet size and pancreatic lipase maximal activity.

occurred, pancreatic lipase, even in the presence of colipase, could no longer penetrate monomolecular films and thus lost its ability to hydrolyze ester linkages.[23] Below such critical values a constant amount of absorbed enzyme was found over a wide range of surface pressures or film composition, but, at the same time the enzyme velocity or the enzyme-specific activity exhibited bell-shaped curves with four-fold variations.[24] In this respect, the data obtained here without lag time suggest that pancreatic lipase can bind and penetrate the mixed monolayer present at the surface of all the emulsions studied, but that the specific activity of the bound enzyme might increase as the curvature of the particle does. This does not mean that curvature per se could be directly involved in the variations observed because the emulsified particles are so large when compared to a lipase molecule. As mentioned above, lipase activation or inactivation occurs at interfaces and these phenomena can be related to surface pressure and to interfacial film composition. We can assume that the particle curvature affects both parameters, but the key factors involved in the modifications of the surface properties remain to be determined.

The higher Vmax observed with the pure MCT emulsion, as compared with the pure LCT emulsion, could be attributed to a size effect (0.19 versus 0.43 μm), but also to a higher enzyme velocity on MCTs, as previously observed with emulsions made in the absence of phospholipids, with pure short-chain, medium-chain, or long-chain triglycerides.[31–33] In that line, it is interesting to compare two emulsions with an almost identical mean droplet size (0.43 and 0.46 μm), but made with either pure LCT or with a 80/20 wt/wt LCT/MCT mixture. The Vm were very comparable (21.3 versus 19.2 units). Thus, although a relative enrichment of the surface monolayer of the mixed emulsion in MCT could be expected,[34] the lipase Vmax was not enhanced. Maybe the presence of low amounts of LCT in the surface monolayer is enough to reduce the mobility of the MCT in phospholipids[34] and thus the hydrolysis rate of MCTs.

In the absence of calcium ions an important lag phase occurred until the maximum lipase activity was reached. A lag phase has been previously described when using emulsions made of tributyrin,[16,28] LCTs, or Intralipid.[15,18] This lag phase was generally interpreted by assuming that an increased lag time means a decreased ability of the enzyme to bind and penetrate the substrate surface. After some experiments,[15,18] excess phospholipids have been proposed to play an important role in the occurrence of the lag phase. Accordingly, it has been demonstrated that pancreatic colipase-lipase can bind to phospholipids dispersed in an aqueous phase.[9,11] Because phospholipid partitioned in the two-phase system between the emulsion surface and the aqueous medium, an increased phospholipid concentration in the aqueous phase might impair colipase-lipase absorption onto the emulsion droplets.[11–13,15] In fact, it is the triglyceride:phospholipid ratio that is related to the surface area concentration, rather than the phospholipid concentration in the aqueous phase, that modulates the exchange of enzyme molecules between the two phases.[12] Depending on experimental conditions, this leads to increased[13] or decreased[12,13,15,16,18] lipase binding and activity. We have already shown that triglyceride:phospholipid weight ratios in the range of 5–15:1 are suitable for a maximum activity of pancreatic lipase on a fine sonicated LCT emulsion.[13] As the four emulsions used in the present study exhibited, with a 9.3:1 triglyceride: phospholipid weight ratio it is unlikely that excess phospholipids per se could hinder pancreatic lipase. This was confirmed by the observation that adding only 1–2 mmol/L calcium was enough to suppress the lag time on both mixed emulsions almost completely. This was in agreement with other studies.[15,18]

Because important lag times were reported in the absence of calcium with very different emulsified systems[15,18,28] or monolayers,[29] it appears that this phenomenon is much more related to a general hindering of the colipase-lipase complex rather than to a particular property of some kind of emulsion. We observed a three-fold higher lag time with the fine MCT/LCT emulsion than with the coarse one. It was suggested that divalent calcium ions enhance the binding and the activity of pancreatic lipase on emulsions by diminishing the electrostatic repulsions occurring between them.[15,22,27,30] This should imply that increasing the concentration of negative charges in the medium requires higher amounts of calcium. This was observed here because 10 mmol/L calcium was necessary to get the maximum lipase activity on the fine emulsion as compared with about 2–4 mmol/L with the coarse emulsion *(Figure 4)*. This calcium concentration ratio is close to that of the calculated mean surface concentration ratio of the fine and coarse MCT/LCT emulsions. In agreement with earlier suggestions,[15,22,27,30] we can assume that calcium ions do counteract negative charges present at the surface of emulsified droplets and that about 20 μmol/m^2 interface of calcium are required to allow a maximum lipase activity (calculated from Lineweaver-Burk plots 1/V f (1/Ca^{++})). Thus, calcium should be added to emulsions in proportion to the surface area. This could have an important significance in some pathological situations where the endogenous calcium supply is restricted, such as in pancreatic insufficiency.

In conclusion, the present study evidences that charging the mean droplet size or the triglyceride composition of complex emulsions, as well as the calcium concentration, can affect the triglyceride hydrolysis catalyzed by pancreatic lipase. This information could be useful to formulate emulsions dedicated to enteral nutrition.

References

1 Mobarhan, S. and Trumbore, L.S. (1991). Enteral tube feeding: a clinical perspective on recent advances. *Nutr. Rev.* **49,** 129–140

2 Carey, M.C., Small, D.M., and Bliss, C.M. (1983). Lipid digestion and absorption. *Ann. Rev. Physiol.* **45,** 651–677

3 Grimble, G., Payne-James, J., Rees, R., and Silk, D. (1988). Physiology of digestion and absorption of enteral diets. *Int. Ther. Clin. Monitor* **8,** 267–274

4 Verger, R. (1984). Pancreatic lipases. In *Lipases,* (B. Borgstrom and H.L. Brockman, eds,) p. 84–150, Elesvier, New York, NY, USA

5 Sarda, L. and Desnuelle, P. (1958). Action de la lipase pancréatique sur les esters en émulsions. *Biochim. Biophys. Acta* **30,** 513–521

6 Hamosh, M. (1990). *Lingual and gastric Lipases: their role in fat digestion,* CRC Press, Boca Raton, FL, USA

7 Benzonana, G. and Desnuelle, P. (1965). Etude cinétique de l'action de la lipase pancréatique sur des triglycérides en émulsion. Essai d'une enzymologie en milieu hétérogène. *Biochim. Biophys. Acta* **105,** 121–136

8 Nalbone, G., Lairon, D., Lafont, H., Domingo, N., Hauton, J., and Sarda, L. (1974). Behavior of biliary phospholipids in intestinal lumen during fat digestion in rat. *Lipids* **9,** 765–770

9 Lairon, D., Nalbone, G., Domingo, N., Lafont, H., Hauton, J., Julien, R., Rathelot, J., Canioni, P., and Sarda, L. (1974). In vitro studies on interaction of rat pancreatic lipase and colipase with biliary lipids. *Lipids* **10,** 262–265

10 Lairon, D., Nalbone, G., Lafont, H., Domingo, N., and Hauton, J.C. (1978). Protective effect of biliary lipids on rat pancreatic lipase and colipase. *Lipids* **13,** 211–216

11 Lairon, D., Nalbone, G., Lafont, H., Leonardi, J., Domingo, N., Hauton, J.C., and Verger, R. (1978). Possible roles of bile lipids and colipase in lipase absorption. *Biochemistry* **17,** 5263–5269

12 Lairon, D., Charbonnier-Augeire M., Nalbone, G., Leonardi, J., Hauton, J.C., Pieroni, G., Ferrato, F., and Verger, R. (1980). The influence of bile salts and bile lipoprotein complex on pancreatic lipase hydrolysis on monomolecular films. *Biochim. Biophys. Acta* **618,** 106–118

13 Lairon, D., Nalbone, G., Lafont, H., Leonardi, J., Vigne, J.L., Chabert, C., Hauton, J.C., and Verger, R. (1980). Effects of bile lipids on the absorption and activity of pancreatic lipase on triacylglycerol emulsions. *Biochim. Biophys. Acta* **618,** 119–128

14 Hernell, O., Staggers, J.E., and Carey, M.C. (1990). Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry* **29,** 2041–2056

15 Borgström, B. (1980). Importance of phospholipids, pancreatic phospholipase A_2, and fatty acid for the digestion of dietary fat. *Gastroenterology* **78,** 954–962

16 Patton, J.S. and Carey, M. (1981). Inhibition of human pancreatic lipase-colipase activity by mixed bile salt-phospholipid micelles. *Am. J. Physiol.* **241,** G328–G336

17 Bläckberg, L., Hernell, O., and Olivecrona, T. (1981). Hydrolysis of human milk fat globules by pancreatic lipase: Role of colipase, phospholipase A_2, and bile salts. *J. Clin. Invest.* **67,** 1748–1752

18 Larsson, A. and Erlanson-Albertsson, C. (1986). Effect of phosphatidylcholine and free fatty acids on the activity of pancreatic lipase-colipase. *Biochim. Biophys. Acta* **876,** 543–550

19 Miller, K.W. and Small D.M. (1982). The phase behavior of triolein, cholesterol, and lecithin emulsions. *J. Colloid Interface. Sci.* **89,** 466–478

20 Nalbone, G., Lairon, D., Charbonnier-Augeire, M., Vigne, J.L., Leonardi, J., Chabert, C., Hauton, J.C., and Verger, R. (1980). Pancreatic phospholipase A_2 hydrolysis of phosphatidylcholines in various physicochemical states. *Biochim. Biophys. Acta* **620,** 612–625

21 Lutz, O., Meraïhi, Z., Ferezou, J., Frey, A., Lutton, C., and Bach, A.C. (1990). The mesophase of parenteral fat emulsion is both substrate and inhibitor of lipoprotein lipase and hepatic lipase. *Metabolism* **39,** 1225–1231

22 Benzonana, G. (1968). Sur le rôle des ions calcium durant l'hydrolyse des triglycérides insolubles par la lipase pancréatique en présence de sels biliaires. *Biochim. Biophys. Acta* **151,** 137–146

23 Verger, R., Rietsch, J., and Desnuelle, P. (1977). Effects of colipase on hydrolysis of monomolecular films by lipase. *J. Biol. Chem.* **252,** 4319–4325

24 Pieroni, G. and Verger, R. (1979). Hydrolysis of mixed monomolecular films of triglyceride/lecithin by pancreatic lipase. *J. Biol. Chem.* **254,** 10090–10094

25 Wieloch, T., Borgström, B., Piéroni, G., Pattus, F., and Verger, R. (1982). Product activation of pancreatic lipase: lipolytic enzymes as probes for lipid/water interfaces. *J. Biol. Chem.* **257,** 11523–11528

26 Kierkels, J.G.T., Vleugels, L.F.W., Kern, J.H.A., Meijer, E.M., and Kloosterman, M. (1990). Lipase kinetics: On-line measurement of the interfacial area of emulsions. *Enzyme Microb. Technol.* **12,** 760–763

27 Scow, R.O. (1988). Effect of sodium taurodeoxycholate, $CaCl_2$, and albumin on the action of pancreatic lipase on droplets of trioleoylglycerol and the release of lipolytic products into aqueous media. *Biochimie* **70,** 1251–1261

28 Borgström, B. (1977). The action of bile salts and other detergents on pancreatic lipase and the interaction with colipase. *Biochim. Biophys. Acta* **488,** 381–391

29 Rietsch, J., Pattus, F., Desnuelle, P., and Verger, R. (1977). Further studies of mode of action of lipolytic enzymes. *J. Biol. Chem.* **252,** 4313–4318

30 Brown, W.J., Belmonte, A.A., and Melius, P. (1977). Effects of divalent cations and sodium taurocholate on pancreatic lipase activity with gum arabic-emulsified tributyrylglycerol substrates. *Biochim. Biophys. Acta* **486,** 313–321

31 Erlanson, C. and Borgström, B. (1970). Tributyrine as a substrate for determination of lipase activity of pancreatic juice and small intestinal content. *Scand. J. Gastroent.* **5,** 293–295

32 Maylié, M.F., Charles, M., Astier, M., and Desnuelle, P. (1973). On porcine pancreatic colipase: Large scale purification and some properties. *Biochem. Biophys. Res. Comm.* **52,** 291–297

33 Brockeroff, H. (1970). Substrate specificity of pancreatic lipase: influence of the structure of fatty acids on the reactivity of esters. *Biochim. Biophys. Acta* **212,** 92–101

34 Deckelbaum, R.J., Hamilton, J.A., Moser, A., Bengtsson-Olivecrona, G., Butbul, E., Carpentier, Y.A., Gutman, A., and Olivecrona, T. (1990). Medium-chain versus long-chain triacylglycerol emulsion hydrolysis by lipoprotein lipase and hepatic lipase: implications for the mechanisms of lipase action. *Biochemistry* **29,** 1136–1142

35 Folch, J., Lees, M., and Stanley, J.H.G. (1957). A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226,** 498–509

36 Amic, J., Lairon, D., and Hauton, J.C. (1972). Technique de dosage automatique de l'orthophosphate de grande fiabilité. *Clinica. Chimica Acta* **40,** 107–114

37 Buccolo, G. and David, H. (1973). Quantitative determination of serum triglycerides by the use of enzymes. *Clin. Chem.* **19,** 476–482

38 Gargouri, Y, Pieroni, G., Riviere, C., Sauniere, J.F., Lowe P.A., Sarda, L., and Verger, R. (1986). Kinetic assay of human gastric lipase on short- and long-chain triacylglycerol emulsions. *Gastroenterology* **91,** 919–925