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Research Article

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The Complete Digestion of Human Milk Triacylglycerol In Vitro Requires Gastric Lipase, Pancreatic Colipase-dependent Lipase, and Bile Salt-stimulated Lipase

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Abstract

Gastric lipase, pancreatic colipase-dependent lipase, and bile salt-stimulated lipase all have potential roles in digestion of human milk triacylglycerol. To reveal the function of each lipase, an in vitro study was carried out with purified lipases and cofactors, and with human milk as substrate. Conditions were chosen to resemble those of the physiologic environment in the gastrointestinal tract of breast-fed infants. Gastric lipase was unique in its ability to initiate hydrolysis of milk triacylglycerol. Activated bile salt-stimulated lipase could not on its own hydrolyze native milk fat globule triacylglycerol, whereas a limited hydrolysis by gastric lipase triggered hydrolysis by bile salt-stimulated lipase.

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fat digestion • breast-feeding • infant nutrition • lipolysis • milk lipase

Introduction

According to the two-phase model of fat digestion and absorption suggested by Hofmann and Borgström, dietary lipids, of which at least 98% are triacylglycerol, are hydrolyzed by colipase-dependent lipase in the proximal part of the small intestine, giving rise to *sn*-2 monoacylglycerols and free fatty acids (FFA).[1] The products of fat digestion are solubilized by bile salts into mixed micelles from which absorption occurs (1, 2). Newborn infants have much lower concentrations of colipase-dependent lipase (3) and bile salts (4, 5) in postprandial duodenal contents than do children or adults. This seems paradoxical since they have a three- to fivefold higher fat intake per kilogram body weight, than the average Western adult. Fat malabsorption does occur in otherwise healthy newborns, especially if they are born before term (6-8). This is, however, a less frequent observation in breast-fed than in formula-fed infants (7, 9). A generally held view is that a major reason for this is the unusual fatty acid distribution of human milk triacylglycerol with palmitic acid preferentially in the *sn*-2 position (10). Since colipase-dependent lipase does not attack the *sn*-2 position (11), most of the palmitic acid is assumed to be absorbed as *sn*-2 monoacylglycerol rather than FFA. It has been advocated that this is beneficial in terms of net fat absorption (12). More recent studies in vitro have however questioned this (9). Furthermore, it has been demonstrated that colipase-dependent lipase on its own does not readily hydrolyze native human milk fat globule triacylglycerols (13-16). Therefore, the efficient utilization of human milk triacylglycerol has been suggested to depend also on gastric lipase and bile salt-stimulated lipase (17).

In this paper we have conducted a study in vitro under conditions attempting to resemble the in vivo gastrointestinal environment of the breast-fed newborn, using human milk triacylglycerol and purified human lipases and cofactors.

Methods

Human gastric lipase was purified from gastric contents (18) collected from a healthy adult male via a naso-gastric tube after subcutaneous injection of pentagastrin (6 μg/kg body wt). Purified human pancreatic colipase-dependent lipase (19) and colipase (20) were generous gifts from Dr. B. Sternby, University of Lund, Sweden. Bile salt-stimulated lipase was purified from human milk as previously described (21).

Taurochenodeoxycholate (≥ 98%, sodium salt) was prepared by Dr. L. Krabisch, University of Lund. Cholate, taurocholate, glycocholate, glycochenodeoxycholate (all ≥ 98%, sodium salts), decanoate, dodecanoate, *n*-9,12-octadecadienate (linoleate), and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO. When indicated, the respective fatty acid was added from a 0.5 mol/liter stock solution in ethanol. Likewise, when indicated, bile salts were added either from a stock solution of 0.2 mol/liter sodium cholate or from a 0.2 mol/liter solution of a physiologic mixture of bile salts (22) containing 84, 44, 52, and 20 mmol/liter of taurocholate, glycocholate, taurochenodeoxycholate, and glycochenodeoxycholate, respectively.

Human milk, collected daily by use of a breast pump from mothers in the maternity ward, was used either fresh or after heat-treatment at 60°C for 15 min to inactivate the milk lipases (23). Milk samples were used only on the day of collection.

Determination of milk triacylglycerol hydrolysis. Unless otherwise stated, lipase activity was determined by continuous titration of the released fatty acids in a pH-stat. Automatic titration was carried out at 37°C with a Metrohm 678 EP/KF Processor, Metrohm 665 Dosimat (Metrohm Ltd., Herisau, Switzerland) with a 1-ml burette containing 20 mmol/liter (Figs. 1-3) or 50 mmol/liter (Fig. 4) NaOH, and a titration assembly (TTA 80; Radiometer A/S, Copenhagen, Denmark). Stirring was set to 1,200 rpm. The standard assay mixture contained 20% (vol/vol) fresh or heat-treated human milk, 5 mmol/liter $CaCl_2$, and 0.15 mol/liter NaCl. pH was set as indicated in legends

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1. *Abbreviation used in this paper:* FFA, free fatty acid.

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to tables and figures, as were additions of enzymes, cofactors, and fatty acids. The amounts of the respective enzymes were chosen to resemble the physiologic situation in intestinal contents of the newborn infant (20, 21, 24). The incubation volume at start was 2 ml (Figs. 1–3) or 5 ml (Fig. 4). When the incubation pH was 6.0, the amount of released fatty acid was calculated from the consumption of NaOH required to reach pH 7.5 relative to a control incubation to which no enzyme was added. This was done to ascertain complete titration of the fatty acids.

In some experiments (Table I) the released fatty acids were extracted and titrated as previously described (16).

Quantitative determination of triacylglycerol, partial acylglycerols, and glycerol. The triacylglycerol concentration of the milk samples used was determined enzymatically by use of a commercial kit (triglycerides without free glycerol; Boehringer Mannheim Gmbh, FRG). Using another kit (glycerol; Boehringer Mannheim), free glycerol was determined in samples taken from the pH-stat reaction mixtures at the times indicated. For determination of triacylglycerol and partial acylglycerols, aliquots were removed from the pH-stat incubation mixtures and boiled in 1% sodium dodecylsulphate for 10 min to inactivate the lipases. This procedure did not affect the recovery of the different lipid classes. The samples (2–20 μl) were run on thin-layer chromatography using Si250-PA(19C) plates (J. T. Baker Chemical Co., Phillipsburg, NJ). The plates were developed first in chloroform/methanol/acetic acid (98:2:0.1, vol/vol/vol). After drying, they were redeveloped in heptane/diethyl ether/acetic acid (94:6:0.2, vol/vol/vol) (25). Chromatographed samples and standards were visualized by charring the plates after staining by $CuSO_4$ (25) and the amount in each spot was determined by reflexion scanning densitometry at 370 nm using a scanning densitometer (Model CS 9000; Shimadzu Scientific Instruments, Inc., Columbia, MD) (26).

Results

Hydrolysis of milk triacylglycerol by bile salt–stimulated lipase. Bile salt–stimulated lipase, a constituent of raw human milk, was on its own unable to hydrolyze native human milk fat globule triacylglycerol (Table I). This was true even in the presence of 5 mmol/liter of activating bile salt, a concentration sufficient for activation (27). However, when the milk triacylglycerol was first hydrolyzed to a limited extent by gastric lipase, continued hydrolysis of the remaining triacylglycerol was then catalyzed by bile salt–stimulated lipase, provided activating bile salt was present (Table I). Purified bile salt–stimulated lipase added to heat-treated milk was as effective as the enzyme in raw milk. The activity obtained between 30 and 60 min of the experiments (Table I) could not be attributed to gastric lipase for two reasons: it was much lower in the absence of bile salt–stimulated lipase (heat-treated milk), and gastric lipase is inhibited at the pH and bile salt concentration used during this part of the incubation (28). The conditions for the experiments in Table I were chosen to be optimal for bile salt–stimulated lipase rather than to resemble physiologic conditions.

Table I. Effects of Gastric Lipase and/or Cholate on the Hydrolysis of Human Milk Triacylglycerol by Bile Salt–stimulated Lipase

			Fatty acids released	
	GL	Cholate	0–30 min	30–60 min
			μmol/ml	
Heat-treated milk	+	−	2.7	0.3
	+	+	2.7	0.7
Raw milk	−	−	0	0
	−	+	0	0.2
	+	−	3.0	0.5
	+	+	3.0	15.5
Heat-treated milk + BSSL	−	+	0	0.4
	+	+	3.1	15.3

The incubation mixture contained 20% (vol/vol) raw or heat-treated human milk, 2% (wt/vol) bovine serum albumin, 50 mmol/liter Tris-maleate buffer (pH 6.0), and 0.15 mol/liter NaCl. Human gastric lipase (GL) (1 μg/ml) and bile salt–stimulated lipase (BSSL) (40 μg/ml) were added at 0 min, when indicated. After 30 min, the incubation pH was shifted to 8.0 by addition of 1 mol/liter Tris-HCl, pH 9.0, and cholate was added to 5 mmol/liter as indicated. At 0, 30, and 60 min, aliquots were withdrawn and released fatty acids extracted and titrated.

In order to resemble the physiologic situation, the activating potential of a mixture of bile salts, physiologic to the newborn, was compared with sodium cholate, the most efficient activating bile salt that we know of (27). The total concentration of bile salt mixture needed to activate bile salt–stimulated lipase (Fig. 1) was within the range found in postprandial duodenal contents of breast-fed newborns (4), although the activity obtained was slightly lower than that obtained with sodium cholate alone. Moreover, when the concentration of the bile salt mixture was increased (compare 2 and 5 mmol/liter) a decreased rate of lipolysis was observed, similar to what has been found previously for individual bile salts in model systems (27). Below 2 mmol/liter bile salt concentration there was a concentration-dependent increase in activity (data not shown). For the following experiments, 2 mmol/liter of the bile salt mixture was chosen.

To study the mechanism responsible for the triggering effect caused by the initial hydrolysis by gastric lipase exogenous

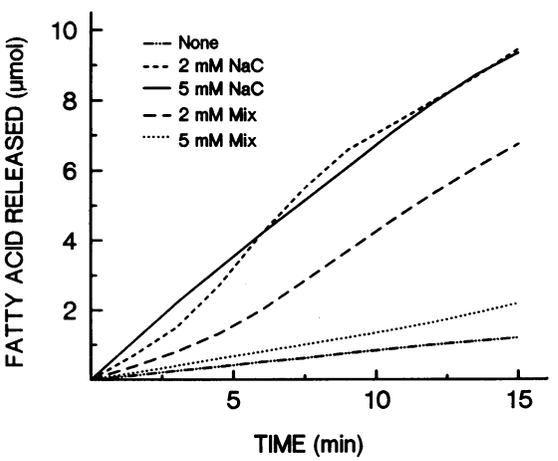

Figure 1. Effects of bile salts on the hydrolysis of human milk triacylglycerol by bile salt–stimulated lipase. Hydrolysis of raw human milk triacylglycerol at the conditions described in Methods was registered in the pH-stat. The milk was preincubated for 10 min with 15 μg/ml gastric lipase at pH 6.0. This resulted in a release of 2.5 μmol fatty acid. At 0 min, bile salt was added and the pH raised to 7.5. The bile salts used were sodium cholate (*NaC*) or the physiologic bile salt mixture (*Mix*). An incubation was also carried out in the absence of bile salts (*None*). 10 μmol of released fatty acid corresponds to hydrolysis of 25% of the ester bonds.

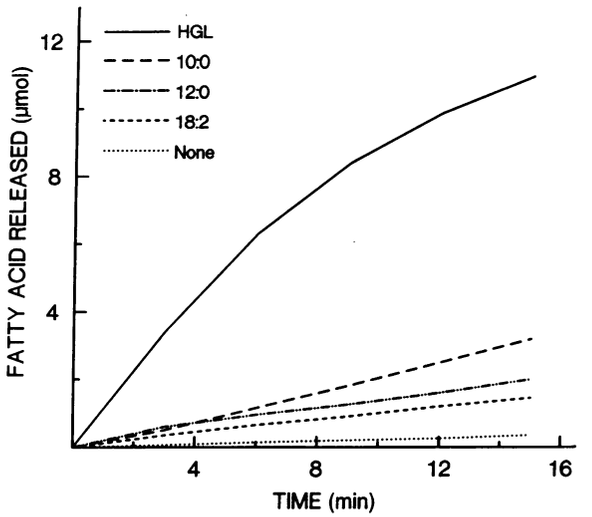

Figure 2. Effects of fatty acids on the hydrolysis of human milk triacylglycerol by bile salt–stimulated lipase. Hydrolysis of raw human milk was followed in the pH-stat at the conditions described in Methods. The incubation mixtures were preincubated for 10 min with 15 μg/ml gastric lipase (*HGL*) or different fatty acids, i.e., 8 mmol/liter linoleate (18:2), 4 mmol/liter decanoate (10:0), or 4 mmol/liter dodecanoate (12:0). The preincubation with gastric lipase resulted in a release of 2.5 μmol fatty acid. At 0 min, the pH was raised from 6.0 to 7.5 and bile salts from the physiologic mixture were added to 2 mmol/liter. 2 μmol released fatty acid corresponds to hydrolysis of 5% of the ester bonds.

FFA were added (Fig. 2). All three fatty acids tested had certain but minor positive effects on the continued lipolysis catalyzed by bile salt–stimulated lipase. The rate of hydrolysis obtained was in no case as high as that obtained after preincubation with gastric lipase. Also, the concentration of exogenously added fatty acid required to obtain initiation of lipolysis was higher than that resulting from hydrolysis by gastric lipase. Addition of lower concentrations of fatty acids resulted in even lower or no hydrolysis.

The effect of preincubation with gastric lipase on bile salt–stimulated lipase-catalyzed hydrolysis was also tested in the absence of bile salts (Fig. 3). Under these conditions, gastric lipase hydrolyzed 20% of the ester bonds in the absence of bile salts. The addition of bile salts and the increase in pH inhibited gastric lipase completely. Bile salt–stimulated lipase was able to continue lipolysis even when no bile salt was present if gastric lipase had hydrolyzed part of the triacylglycerols (Fig. 3).

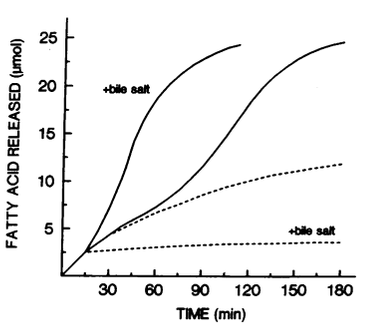

Figure 3. Effect of gastric lipase on the hydrolysis of human milk triacylglycerol by bile salt–stimulated lipase in the absence of bile salts. Hydrolysis of heat-treated (*dotted lines*) or raw (*solid lines*) human milk triacylglycerol was followed in the pH-stat as described in Methods. At 0 min, 6 μg/ml gastric lipase was added. After 15 min, the pH was raised from 6.0 to 7.5 and bile salts (physiologic mixture) were added to 2 mmol/liter as indicated. The release of 10 μmol of fatty acid corresponds to hydrolysis of 25% of the ester bonds.

Sequential hydrolysis of milk triacylglycerol catalyzed by combined effects of the three lipases. In order to reveal the overall mechanisms of milk triacylglycerol digestion, we studied the rate and extent of lipolysis catalyzed by the three relevant lipases under conditions assumed to resemble their physiologic environment with regard to dietary lipid substrate, pH, bile salt composition and concentration, lipase, and cofactor concentrations (Fig. 4). Earlier studies have shown that gastric lipase was important for subsequent hydrolysis by colipase-dependent pancreatic lipase (16, 29, 30). The compositional analysis of the reaction mixture after the initial incubation with gastric lipase revealed that low concentrations of mainly FFA and diacylglycerol had been formed (Fig. 4 *B, a*). Colipase-dependent lipase continued lipolysis so that about two thirds of the total ester bonds were hydrolyzed, giving rise chiefly to FFA and monoacylglycerols (Fig. 4 *A, b* and *B, b*). This product pattern remained virtually unchanged even after prolonged incubation (Fig. 4 *B, d*). Addition of bile salt–stimulated lipase at a point (110 min) where colipase-dependent lipase caused little further lipolysis (Fig. 4 *A, b*) had profound effects on the degree of lipolysis (Fig. 4 *A, c*), as well as on the product composition (Fig. 4 *B, c*). More than 95% of the total ester bonds were hydrolyzed under these conditions, and FFA

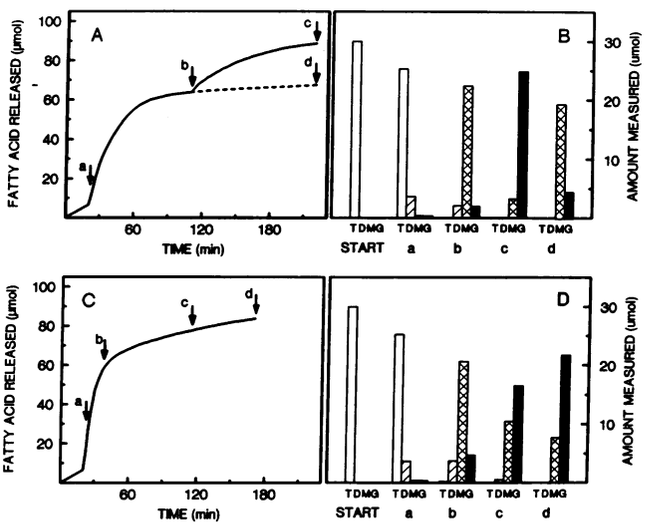

Figure 4. Hydrolysis of human milk triacylglycerol by gastric lipase, colipase-dependent lipase, and bile salt–stimulated lipase. Hydrolysis of heat-treated human milk triacylglycerol was followed in the pH-stat as described in Methods. At 0 min, 6 μg/ml gastric lipase was added. After 20 min, the pH was raised from 6.0 to 7.5 and bile salts (physiologic mixture) were added to 2 mmol/liter followed by the addition of colipase-dependent lipase (2.5 μg/ml) and colipase (0.5 μg/ml). In *A*, after 115 min, 40 μg/ml bile salt–stimulated lipase was added to one incubation (*solid line*) and no further addition was made to the other (*broken line*). In *C*, bile salt–stimulated lipase (40 μg/ml) was included from start (0 min). Hydrolysis of 100% of the ester bonds corresponds to the release of 96 μmol fatty acid. At the start of the incubation and at the times indicated by the arrows (*a–d*) samples were withdrawn for determination of the product pattern. *B* and *D* show the decrease in relative concentration of triacylglycerol (T) and the increase with time of diacylglycerol (D), monoacylglycerol (M), and glycerol (G) in the incubations of *A* and *C*, respectively.

and glycerol became the predominant products. The molar amount of free glycerol formed corresponded to > 90% of that originally bound as milk triacylglycerol (Fig. 4 *B*). In order to more closely resemble the situation in duodenal contents of the breast-fed newborn, similar incubations were carried out but with colipase-dependent lipase and bile salt–stimulated lipase acting simultaneously (Fig. 4 *C*). This resulted in a higher initial rate of lipolysis than for colipase-dependent lipase alone (compare *A* and *C* in Fig. 4). As expected, the extent of lipolysis was increased as compared with that obtained for colipase-dependent lipase alone (compare *A, d* and *C, d* in Fig. 4). The product pattern of the initial rapid phase resembled that obtained by colipase-dependent lipase alone, illustrating that bile salt–stimulated lipase also contributed to hydrolysis of tri- and diacylglycerol. The amount of free glycerol was, however, increased already at early time points. As in the case of sequential addition of the enzymes (Fig. 4 *B*), with time glycerol and FFA became the major products also when the two enzymes were operating simultaneously (Fig. 4 *D*).

Discussion

Human milk fat is secreted as globules; the triacylglycerol, accounting for ~ 98% of total lipids, constitutes the core of the globule which when extruded from the epithelial cell becomes enveloped, and thus stabilized by the apical part of the plasma membrane (31). These surface components, mainly phospholipid and protein, known as the milk fat globule membrane, have profound effects on the accessibility of the triacylglycerol for lipase-catalyzed hydrolysis. In fact, pancreatic colipase-dependent lipase is on its own virtually without activity against native human milk globule triacylglycerol (13, 14, 16). Similar observations have been made with synthetic triacylglycerol emulsion particles covered with phospholipid or dietary proteins such as β-lactoglobulin, lactoferrin, and albumin (29, 32–34). It seems as if the presence of other surface active components denies the lipase access to the triacylglycerol. There are however mechanisms by which the inhibition of colipase-dependent lipase can be reduced, i.e., addition of colipase, of phospholipase A_2, and of bile salts (14, 15, 33, 34). However, studies using either an artificial phospholipid-covered emulsion (Intralipid) or human milk as substrate have shown that when added in physiologic concentrations neither of these factors alone is enough to effectively relieve the inhibition (16, 29). Recent observations suggest that FFA might be the most important lipolysis-promoting factor. Disregarding their source, i.e., generated endogenously by gastric lipase (16, 29), or another similar lipase (33), or by phospholipase A_2 (34, 35), or being added as exogenous FFA (16, 29), they will initiate lipolysis by colipase-dependent lipase, provided colipase and bile salt are present (16). One important effect seems to be that they cause increased binding of the lipase to the substrate droplets (16, 34).

Among the gastrointestinal lipases, gastric lipase is unique in its ability to attack native human milk fat globules (16, 17, 36). By analogy, it readily hydrolyzes an artificial emulsion also when the surface is covered by phospholipid and/or proteins such as β-lactoglobulin and albumin (37). Inasmuch as gastric lipase is inhibited by fatty acids (16, 29), it seems unlikely that its most important function is to make a major quantitative contribution to lipid digestion. Furthermore, bile salts inhibit gastric lipase when using human milk (Fig. 3) (28) or a synthetic emulsion of long-chain triacylglycerol (38) as substrate which will make lipolysis in the duodenum due to gastric lipase unlikely. The combination of gastric lipase and colipase-dependent lipase will, however, hydrolyze as much as two thirds of the ester bonds, resulting in FFA and *sn*-2 monoacylglycerol as end products. These are considered the essential steps of triacylglycerol digestion in adults (2, 39).

It has repeatedly been shown that human milk triacylglycerol is more efficiently utilized than triacylglycerol from cow's milk–based formulae (7–9, 40). A likely explanation is the presence of bile salt–stimulated lipase in human milk (41, 42). Pasteurization, which inactivates the milk lipase, reduces fat absorption from human milk by approximately one third (8, 43). Bile salt–stimulated lipase resists the conditions of gastric contents and is present in an active form in duodenal contents (24).

There was a complete analogy between colipase-dependent lipase and bile salt–stimulated lipase inasmuch as the latter showed little activity against native human milk triacylglycerol even in the presence of activating bile salt. The activity of bile salt–stimulated lipase was efficiently triggered by a limited lipolysis catalyzed by gastric lipase. In contrast to colipase-dependent lipase (16, 29, 34), the triggering effect could not be fully mimicked by the addition of fatty acids (Fig. 2). It is possible that partial acylglycerol generated by gastric lipase was even more important. This is supported by the unexpected finding that bile salt–stimulated lipase could hydrolyze partly digested milk triacylglycerol even in the absence of bile salts (Fig. 3). It has been shown previously that the enzyme hydrolyzes at least monoacylglycerol in the absence of activating bile salt (44). In this context, it should be noted that the concerted action of gastric lipase and bile salt–stimulated lipase represents a route for fat digestion unique to the breast-fed infant, which should be of particular importance under pathological conditions such as cystic fibrosis or other causes of pancreatic insufficiency.

The most important observation was that under the conditions used, chosen to resemble the physiologic situation, human milk triacylglycerol digestion seem to be a process catalyzed by all three lipases: gastric lipase, colipase-dependent lipase, and bile salt–stimulated lipase (Fig. 4). Each lipase contributes with its specific properties. In contrast to colipase-dependent lipase, bile salt–stimulated lipase hydrolyzes also *sn*-2 monoacylglycerol (44–46). Hence, when the milk enzyme was added to an incubation where human milk triacylglycerol had been hydrolyzed extensively by gastric lipase and colipase-dependent lipase so that no further net lipolysis occurred, lipolysis recommenced due to hydrolysis of the monoacylglycerol by bile salt–stimulated lipase. Thus, although fatty acids were not removed by absorption as they would be in the physiologic situation, virtually all ester bonds were hydrolyzed to FFA and glycerol. This contradicts the view that the high coefficient of fat absorption from human milk is explained by palmitic acid being esterified preferentially to the *sn*-2 position of human milk triacylglycerols (12). In the breast-fed infant, the palmitic acid in the *sn*-2 position will be released and therefore absorbed as FFA. This may in fact be beneficial to the newborn infant because there is ample evidence that fatty acids are more readily absorbed than monoacylglycerol under conditions of low intraluminal levels of bile salts (47, 48).

When the milk enzyme and the colipase-dependent lipase

acted simultaneously, the former also affected the initial rate of lipolysis (Fig. 4 *C*). The addition of bile salt–stimulated lipase doubled the initial rate (compare *A* and *C* in Fig. 4). This is in agreement with previous studies that have shown in some infants that half the lipase activity found in duodenal contents after a meal of raw human milk was attributed to the milk enzyme (24). A dual function of bile salt–stimulated lipase is implicated. In infants with comparatively high intraduodenal bile salt concentrations, i.e., individuals with high capacity of micellar solubilization, transport, and absorption of monoacylglycerol (49), bile salt–stimulated lipase assists colipase-dependent lipase in the hydrolysis of tri- and diacylglycerol. This is supported by the observation that it hydrolyzes tri- and diacylglycerol at higher rates than monoacylglycerol (50). If, however, the bile salt concentration is low, as in many preterm infants, solubilization and absorption of monoacylglycerol is hampered, which allows the milk enzyme to hydrolyze monoacylglycerol. A dual function of bile salt–stimulated lipase is further supported by the fact that in breast-fed preterm infants there was no correlation between the intraduodenal bile salt levels and the coefficient of fat absorption, whereas there was such a correlation when fed cow's milk–based formula devoid of bile salt–stimulated lipase (22, 51).

In conclusion, efficient digestion of human milk triacylglycerol in vitro depends on the concerted action of three lipolytic enzymes: gastric lipase, colipase-dependent lipase, and bile salt–stimulated lipase. Each enzyme has its unique, only partly overlapping, function. The results are compatible with a view that in infants with low intraluminal bile salt concentrations, their concerted action will result in complete digestion of the triacylglycerol so that FFA and free glycerol become the end products to be absorbed by the intestinal mucosa. Recent studies strongly suggest that under these conditions absorption of fatty acid may well occur chiefly from unilamellar vesicles rather than from mixed micelles (52, 53).

Acknowledgments

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