

# Ontogeny and chain-length specificity of gastrointestinal lipases affect medium-chain triacylglycerol utilization by newborn pigs<sup>1</sup>

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**ABSTRACT:** Ontogeny and fatty acid chain-length specificity of gastrointestinal lipases in neonatal piglets were examined to explore the basis for variations in postnatal use of medium-chain triacylglycerols (MCT). Twenty-four newborn pigs were studied at 4 ages: 0, 6, 18, and 48 h postpartum (n = 6 pigs/age). Piglets were gastrically intubated and given 3.0 mmol/kg of BW<sup>0.75</sup> each of emulsified tri-C6:0 and tri-C8:0. One hour after intubation, the plasma concentration of C6:0 was 7.5-fold greater than that of C8:0 ( $P < 0.001$ ), with total plasma medium-chain fatty acid concentrations 3.7-fold greater at 48 h than at 6 h of age ( $P < 0.05$ ). Pancreatic, gastric, and lingual tissues were analyzed for lipase activity using an equimolar mixture of tri-C6:0 and tri-C8:0 as substrate. Pancreatic lipase activity averaged  $7.0 \pm 0.8$   $\mu\text{mol}$  of fatty acid released/min per mg of protein for the medium-chain fatty acid substrates.

Hexanoate (C6:0) release was greater at 0 h than at 6, 18, or 48 h ( $P < 0.05$ ); however, age did not affect C8:0 release ( $P > 0.05$ ). The lowest lipase activity was observed at 18 h for both tri-C6:0 and tri-C8:0. Chain-length specificity of pancreatic lipase was measured with tri-C4:0, tri-C6:0, tri-C8:0, and tri-C10:0 as combined or separate substrates. As separate substrates, the lipase activity decreased progressively as chain length increased from tri-C4:0 to tri-C10:0. As combined substrates, tri-C6:0 was hydrolyzed fastest ( $P < 0.05$ ), followed by C4:0, C8:0, and C10:0. Gastric and lingual lipase activities averaged 2.7 nmol/min per mg of protein for the medium-chain fatty acid substrates, with hydrolysis of C6:0 being 7-fold greater than that of C8:0. In conclusion, pancreatic lipase dominates the preduodenal lipases in the neonatal pig, and greater activity of the gastrointestinal lipases toward tri-C6:0 underlies its increased rate of use.

**Key words:** fat digestion, lipase, medium-chain triacylglycerol, ontogeny, pig

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## INTRODUCTION

Neonates of most mammalian species must rapidly adapt to an abrupt change in fuel supply; from predominantly carbohydrate provided in utero to predominantly lipid provided postnatally via milk (Battaglia and Meschia, 1985). Thus, rapid postnatal development of lipid digestion is critical for neonates to thrive. Infants born prematurely demonstrate reduced fat digestibility, which can be improved by enrichment of formulas with medium-chain triacylglycerols (MCT) that are more rapidly and completely digested than typical milk lipids (Bach and Babayan, 1982).

Our laboratory has extensively examined the use of MCT using a neonatal pig model (Odle, 1997), with results applicable both to biomedicine and agriculture. However, major gaps in our knowledge still exist, especially in the early postnatal period. Specifically, the ontogeny of the gastrointestinal lipases has not been systematically measured before 1 wk of age, which constitutes an important period in development, given that most piglet deaths occur within 7 d of birth (USDA, 2002). Furthermore, previous research has shown marked effects of fatty acid chain length and emulsification on the rate and extent of MCT use by piglets (Odle, 1997). Indeed, 1 h after oral gavage of tri-C7:0, plasma C7:0 concentrations were 50-fold greater than C10:0 concentrations in pigs gavaged with tri-C10:0 (Odle et al., 1991). Wieland et al. (1993a,b) extended these findings to chain lengths as low as to C4:0, gavaging MCT in both emulsified and nonemulsified states. Emulsified tri-C6:0 produced the greatest plasma fatty acid concentrations, which were 2- to 50-fold greater than tri-C8:0 or tri-C10:0 (Wieland et al., 1993a; Odle et al., 1994). We also showed that the rate of radiola-

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beled tri-C8 use (digestion, absorption, and oxidation) vastly exceeded that of tri-C18:1, and that supplemental carnitine was without effect (Heo et al., 2002).

Therefore, the research described herein was designed to determine how the ontogeny and chain-length specificity of the gastrointestinal lipases influence MCT use by the neonatal pig.

## MATERIALS AND METHODS

### *Animal Protocol and Tissue Collection*

All animal procedures were approved by the University of Illinois Laboratory Animal Care Committee. Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Twenty-four neonatal piglets, ranging in weight from 1.0 to 1.9 kg, were used in the study. Pregnant sows fed a standard corn-soybean meal diet were acquired at the University of Illinois Swine Research Center. Three sows were allowed to farrow naturally after being moved into the farrowing facilities on d 109 of gestation. Eight piglets from each of the 3 litters were randomly allotted to 4 treatment ages: 0, 6, 18, and 48 h postpartum. The exact time of birth was recorded for each piglet and used as its starting time. Except for 0-h animals, piglets were allowed to remain with and suckle their dam until tissue collection.

Colostrum-deprived 0-h piglets were immediately removed from the sow, weighed, and gastrically intubated to administer 3.0 mmol/kg of BW<sup>0.75</sup> each of tri-C6:0 and tri-C8:0 (>98% purity; Karlshamns Lipid Specialties, Columbus, OH) as 30% oil-in-water emulsions containing 2% (wt/vol) Tween-80 in the aqueous phase (Wieland et al., 1993b). All of the other piglets were gavaged with MCT emulsions after food deprivation for 1 h before their respective treatment age. During this time and for an additional 1 h after intubation, animals were individually housed at 30°C. At 1 h postgavage, animals were killed by electrocution (Am. Vet. Med. Assoc.-approved procedure), and blood samples were collected by cardiac puncture using EDTA as anticoagulant. Blood was centrifuged at 4°C and 8,000 × g, and the plasma was stored at -70°C.

The pancreas and tongue (including the most posterior portions) were immediately removed, weighed, and frozen in liquid nitrogen. The stomach was removed, flushed free of digesta, weighed, and stored on ice for immediate homogenization. Tissues were homogenized on ice, using a Brinkman Polytron tissue homogenizer (Kinematica, Luzern, Switzerland). The stomach and tongue were homogenized (1:4) in an electrolyte solution (150 mM NaCl; 6 mM CaCl<sub>2</sub>), whereas the pancreas was homogenized in distilled, deionized water to form a 20% homogenate. Homogenates were centrifuged for 20 min at 4°C and 12,000 × g, and the translucent supernatants were decanted, and stored at -70°C.

### *Medium-Chain Fatty Acid Analysis*

Plasma medium-chain fatty acids were extracted, derivatized to phenacyl esters, and quantified by HPLC (Odle et al., 1991; Wieland et al., 1993a) using heptanoate as an internal standard.

### *Gastrointestinal Lipase Analyses*

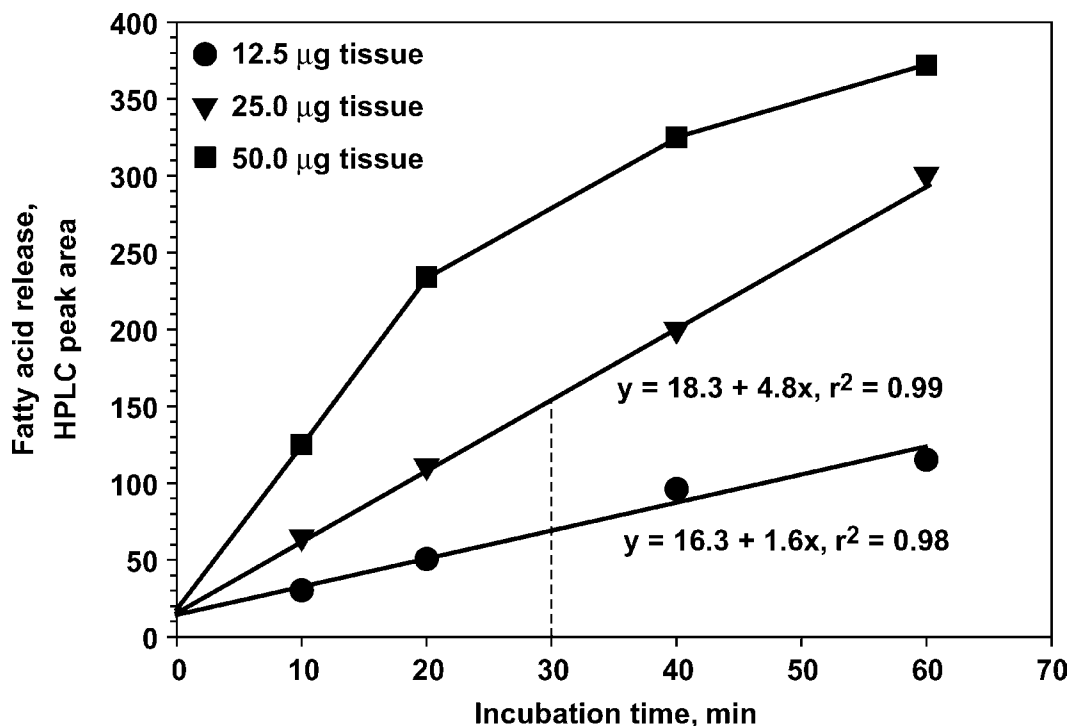
Assays were patterned after those described previously (DeNigris et al., 1988; Armand et al., 1992). It should be noted that the assay conditions were optimized for medium-chain triglyceride emulsions and empirically determined to maintain assay linearity (described below), but the resultant activity measurements do not necessarily represent V<sub>max</sub> rates nor do they apply to other (long-chain) substrates.

Pancreatic tissue homogenate (50 μL, 10 μg of tissue) was mixed with 90 μL of electrolytes (150 mM NaCl, 3.8 mM CaCl<sub>2</sub>), 60 μL of taurocholic acid solution (28.8 mM), 100 μL of phosphate buffer (100 mM, pH 8.0; with 0.015 mM BSA), 50 μL of porcine colipase (0.25 μg/mL), and 0.12 g of triacylglycerol lecithin emulsion (composed of 0.1 mmol each of tri-C6:0 and tri-C8:0, 4.1 mg of glycerol, and 11.7 mg of L-α-phosphatidylcholine) in a 16 × 100 mm disposable culture tube (Fisher Scientific, Pittsburgh, PA), and incubated for 30 min at 37°C, with constant shaking. The reaction was stopped by addition of 2 mL of chloroform/MeOH (1:2, vol/vol) prechilled to 4°C. Heptanoate (C7:0; 25 μL, 8.2 mM) was added to each reaction mixture as an internal standard. Fatty acid extraction, derivatization to phenacylestes, and quantitation by HPLC were as described for plasma.

The efficacy of the pancreatic lipase assay was demonstrated by linearity of blank-corrected fatty acid release over 60 min at several tissue concentrations (Figure 1). Based on these results, we chose to assay ~10 μg of homogenate with an incubation time of 30 min (not to exceed 200 area units) to insure linearity.

Pancreatic chain-length specificity was measured by the same methods described above, with the following exceptions. Two types of substrates were used; in one, the substrates (0.05 mmol each of tri-C4:0, tri-C6:0, tri-C8:0, and tri-C10:0) were combined in a lecithin emulsion (4.1 mg of glycerol and 11.7 mg of phosphatidylcholine) and assayed simultaneously; in the other, the substrates were emulsified separately and assayed individually. The total triacylglycerol concentration was constant in each assay, in that the molar amount of each triacylglycerol in the combined-substrate assay was one-fourth the molar amount in the individual-substrate assays. Activity was measured using pancreatic tissue homogenates from three 6-h-old piglets.

Gastric tissue homogenates (150 μL, 30 μg of tissue in 150 mM NaCl with 6 μM CaCl<sub>2</sub>) were mixed with buffer (300 μL; 50 mM succinate, pH 5.8, with 0.015 mM BSA) and other components as described for pancreatic lipase. The reaction parameters for the lingual lipase



**Figure 1.** Time course of medium-chain triacylglycerol hydrolysis by pancreatic lipase. Medium-chain fatty acid release (HPLC peak area) was measured after 10, 20, 40, and 60 min of incubation at 37°C with 12.5, 25, or 50 µg of pancreatic tissue. Regression equations are indicated for the lower tissue concentrations. Subsequent assays were conducted with ~10 µg of fresh tissue over a fixed interval of 30 min (vertical reference line), and were used only if the peak area was less than 200 units.

assay were similar except that 200 µL of lingual tissue (40 µg of tissue in 150 mM NaCl; 6 mM CaCl<sub>2</sub>) was used.

Tissue homogenate protein concentrations were determined using the biuret method, as described by Gornall et al. (1949).

### Statistical Analyses

Data were subjected to 2-way ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC) appropriate for a split-plot design (Steel and Torrie, 1980), with whole-plot factors consisting of litter (blocking variable) and piglet age, and the subplot consisting of fatty acid (C6:0 vs. C8:0), and the age × fatty acid interaction. The detailed assessment of chain-length effects was evaluated using 1-way ANOVA. In each case, means were compared using a protected-LSD test (Steel and Torrie, 1980) and were considered different when  $P < 0.5$ .

## RESULTS

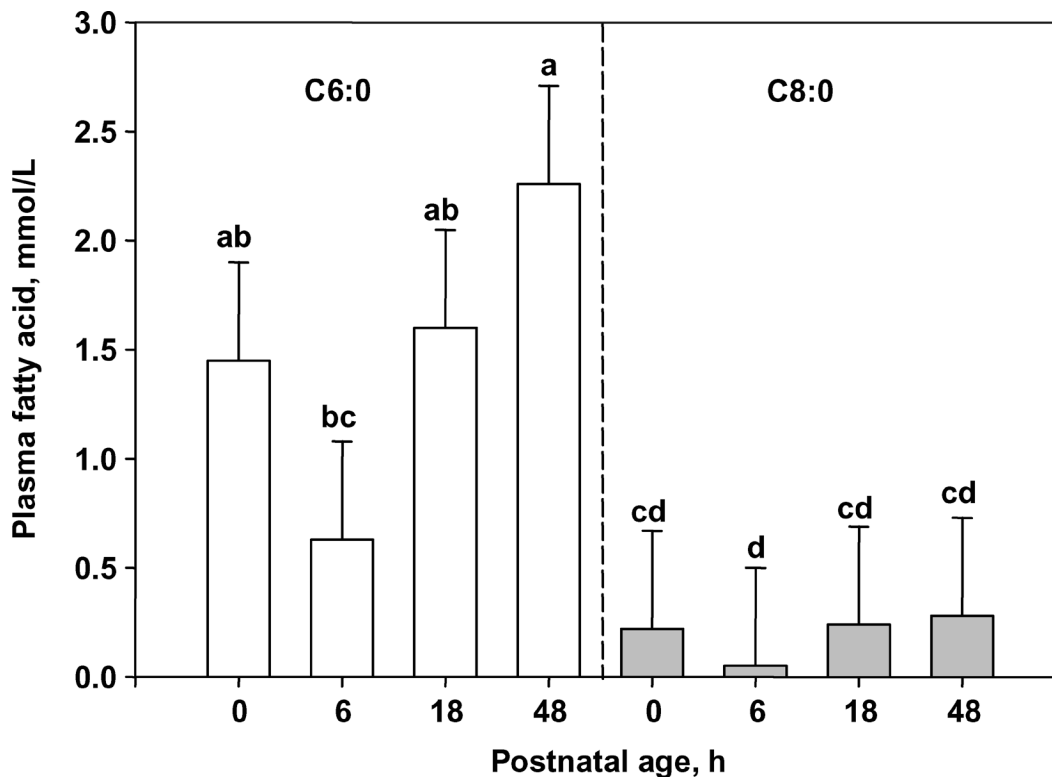
### Plasma Fatty Acid Concentrations

Plasma C6:0 concentrations in piglets given the equimolar mixture of tri-C6:0 and tri-C8:0 greatly exceeded C8:0 concentrations at all ages ( $P < 0.05$ ), averaging 7.5-

fold greater overall (Figure 2). Both fatty acid profiles followed a similar pattern with a dip in concentration at 6 h followed by a rebound at 18 h; however, changes in C8:0 concentration were not detected ( $P = 0.46$ ). The concentration of C6:0 in 48-h-old pigs rose further and was more than 3-fold greater than concentrations in 6-h-old pigs ( $P < 0.05$ ).

### Ontogeny of Pancreatic Lipase

Pancreatic lipase specific activity (Figure 3) measured using a mixed equimolar emulsion of tri-C6:0 and tri-C8:0 was greatest (17.1 and 3.4 µmol fatty acid released/min per mg of protein for C6:0 and C8:0, respectively) in newborn pigs (0 h;  $P < 0.05$ ). Because we homogenized and assayed pancreatic tissue directly (rather than luminal contents), these data reflect stored lipase activity rather than “secretory activity.” Activity steadily decreased to the lowest levels (6.5 and 1.0 µmol/min per mg of protein for C6:0 and C8:0) in 18-h-old pigs, and then the rate of C6:0 release rebounded in 48-h-old pigs. This pattern of activity was observed for both C6:0 and C8:0, although hydrolysis of tri-C6:0 was 5-fold greater on average than that of tri-C8:0 ( $P < 0.001$ ). The release of C8:0 was not different at the 4 ages ( $P = 0.23$ ), resulting in an age × fatty acid chain-length interaction ( $P < 0.05$ ). Total lipolytic capacity of the pancreas [scaled to piglet BW (i.e., µmol/min per



**Figure 2.** Plasma hexanoate (C6:0) and octanoate (C8:0) concentrations in piglets 1 h after gastric intubation and administration of medium-chain triacylglycerol. At each postnatal age (0, 6, 18, and 48 h), piglets were given 3.0 mmol of tri-C6:0 and 3.0 mmol of tri-C8:0 per kg of BW<sup>0.75</sup> as a 30% (vol/vol) emulsion. <sup>a-d</sup>Means  $\pm$  SEM (n = 6) lacking a common superscript letter are different ( $P < 0.05$ ).

kg of BW)] showed a similar age distribution except that the rebound in 48-h-old pigs yielded the greatest overall capacity for tri-C6:0 hydrolysis ( $P < 0.05$ ; data not shown).

#### Gastrointestinal Lipase Activity Comparison

Gastric and lingual lipase specific activities were very low, approaching the detection limit by HPLC. Furthermore, because activities were of similar magnitude at all ages (data not shown), only overall averages are presented. Mean gastric lipase specific activity was 2.7 nmol/min per mg of protein and lingual lipase activity was 1.8 nmol/min per mg of protein. On average, C8:0 release rate was 14% of that for C6:0 (data not shown). Computation of total organ lipolytic capacities (Figure 4) showed that pancreatic capacity exceeded the combined capacities of gastric and lingual tissues by more than 1,000-fold.

#### Chain-Length Specificity of Pancreatic Lipase

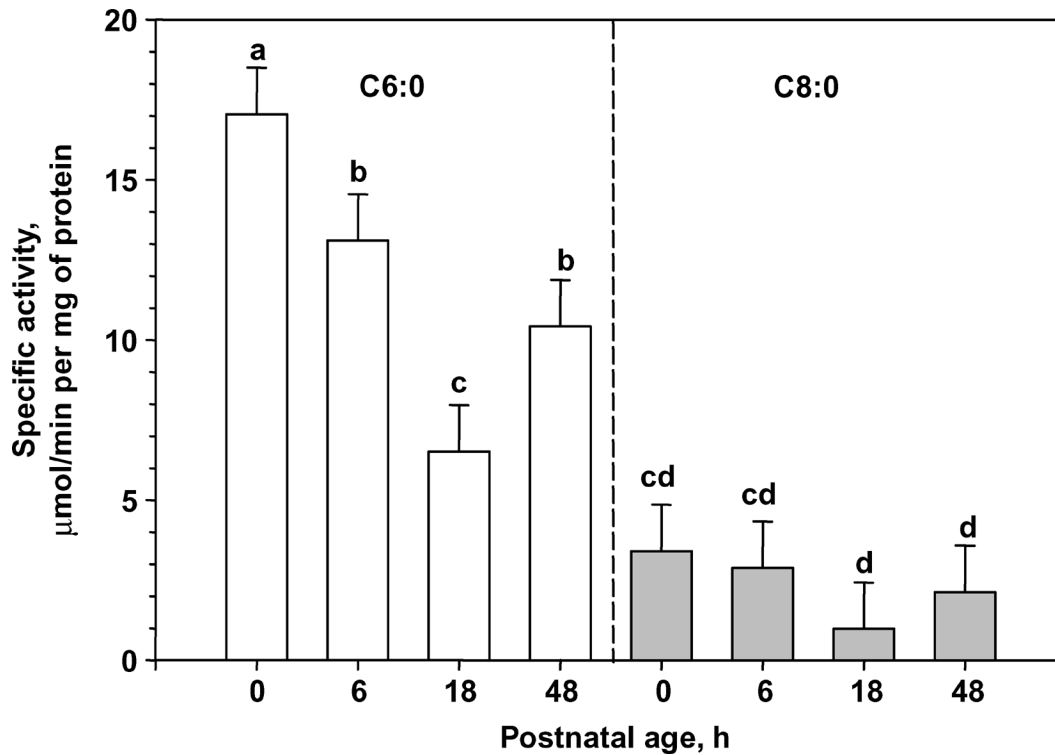
When activity was measured on individual tri-C4:0 through tri-C10 substrates separately (Figure 5), the rates were similar for C4:0, C6:0, and C8:0 (downward tendency), but were an order of magnitude less for C10:0 ( $P < 0.05$ ). When the 4 chain lengths (4, 6, 8, and 10) were assayed as a single combined equimolar

emulsion, tri-C6:0 was hydrolyzed at the greatest rate, followed by C4:0, C8:0, and C10:0, although only the values for C6:0 and C10:0 were different ( $P < 0.05$ ); activity toward tri-C10:0 represented 9% of the activity toward tri-C6:0.

## DISCUSSION

#### Lipase Ontogeny

The transient decrease in MCT use inferred from diminished plasma medium-chain fatty acid concentrations in pigs gavaged at 6 h of age was paralleled by a drop in specific activity of pancreatic lipase, which continued to decline through 18 h before rebounding in 48-h-old piglets. This small discordance in the time course of plasma fatty acid concentration vs. lipase activity would be expected if the lipase measured in the pancreas does not correlate directly with lipase present in the duodenal lumen. Tissue lipase content is a function of its rate of synthesis and its rate of secretion. At birth, accumulation and storage of lipase appears to be relatively high, suggesting synthesis with limited secretion during the prenatal period. In the early postnatal period, if secretion rate exceeds synthesis rate, then tissue levels would rapidly decrease (as observed). Indeed, it would appear that this is the case for the first 18 h; thereafter, synthesis may exceed secretion.



**Figure 3.** Specific activity of pancreatic lipase as a function of piglet age and fatty acid chain length. Hydrolysis rate of an equimolar emulsion of tri-C6:0 and tri-C8:0 (200 mM each) was assayed at each age (0, 6, 18, and 48 h). Activity was expressed as micromoles of fatty acid liberated per minute per milligram of pancreatic tissue protein. <sup>a-d</sup>Means  $\pm$  SEM (n = 6) lacking a common superscript letter are different ( $P < 0.05$ ).

Our findings complement and extend previous reports of pancreatic lipase ontogeny. According to Rokos et al. (1963), pancreatic lipase specific activity in neonatal rats rapidly decreased after birth and continued to be low until weaning. Kitts et al. (1956) measured pancreatic lipase activity in the preweaning piglet at 0, 7, 14, 21, 35, and 48 d of age. They found activity to be low at birth, followed by a steady increase in activity between 7 and 14 d, and plateauing around d 48. Corring et al. (1978) also examined pancreatic lipase ontogeny at weekly intervals in the suckling pig and found a marked increase in activity between the third and fourth weeks of age and a smaller increase between the fourth and eighth weeks.

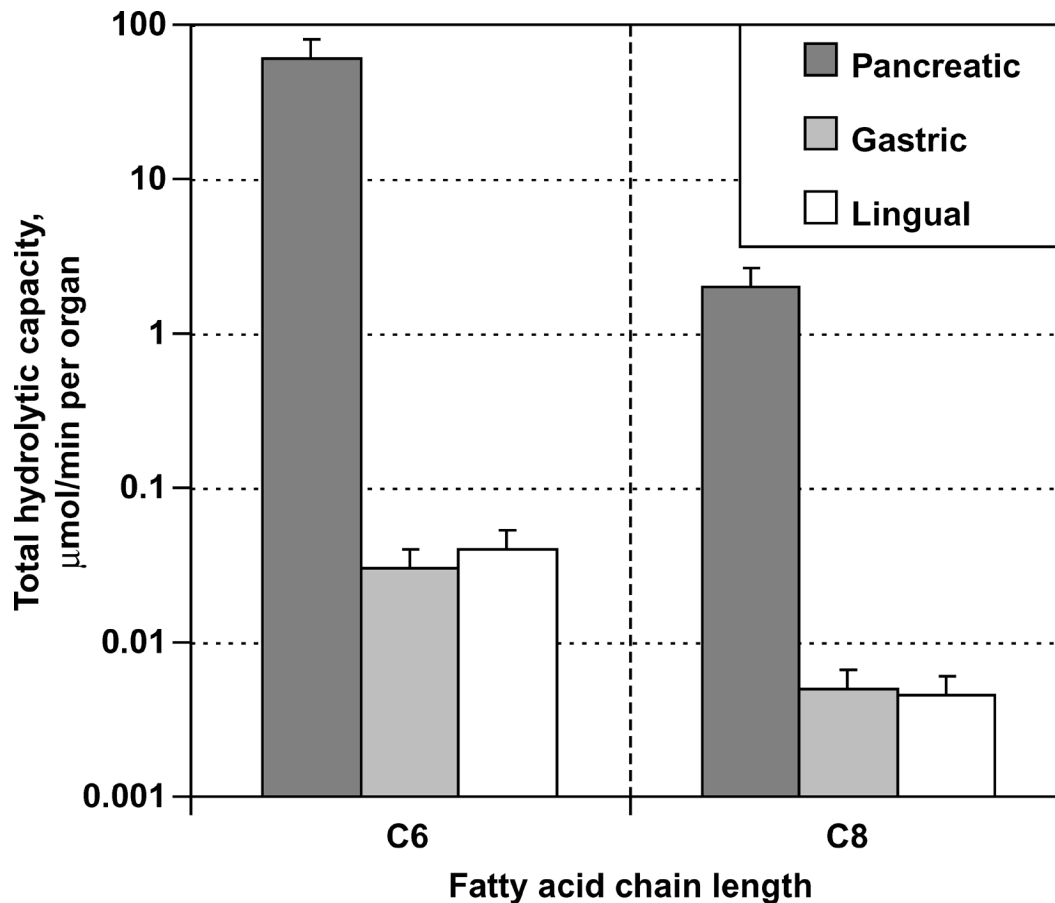
Pancreatic lipase related proteins (Payne et al., 1994), if present, might account for some of the observed lipase activity, and could explain the greater initial activity (0 h vs. 18 h). In rats, mRNA for pancreatic lipase related proteins increased prenatally to reach its maximal abundance 12 h postpartum, and then declined to low levels in the adult animal (Payne et al., 1994). Similarly, the consumption of milk fat by suckling neonates may itself signal increased expression of pancreatic lipase (Wicker-Planquart and Puigserver, 1993).

### *Preduodenal Lipases*

Preduodenal lipases are estimated to hydrolyze 10 to 30% of the triacylglycerols before the small intestine

in some species (Hamosh, 1990). These lipases are characterized to have broad pH profiles, spanning from 3 to 6.5, thus allowing activity in both the stomach and the intestine. From this, the expectation might be that lingual and gastric lipase activities are within an order of magnitude of pancreatic lipase in the young animal. However, this was not evident in our findings. Pancreatic lipase activity was 3 orders of magnitude greater than the combined preduodenal lipases. Thus, we infer that preduodenal lipases do not appear to contribute substantially to lipolysis in the early postnatal period of the piglet. Moreau et al. (1988) found only a minimal amount of lingual lipase activity in adult swine, compared with the amount of gastric lipase. Lingual lipase accounted for 7.1% of the total preduodenal lipase in adult pigs, whereas, in neonatal piglets, the mean lingual lipase activity was nearly equal to that of gastric lipase. Similarly, Jensen et al. (1997) found that pancreatic lipase capacity greatly exceeded gastric lipase of pigs from 3 to 56 d of age.

Perinatal rat lingual lipase activity significantly increases 2 to 3 d before the end of gestation (Hamosh and Hand, 1978). After parturition and the first suckling period (within the first 6 h), lingual lipase specific activity decreased by 50%. By d 2, lipase specific activity had returned to (or surpassed) the activity measured at birth, and continued to increase sharply thereafter. This decrease was theorized to be a function of stored enzyme release. Ontogeny of other enzymes, such as



**Figure 4.** Total gastric, lingual, and pancreatic lipase activity in newborn pigs (log scale) averaged over postnatal age (0, 6, 18, and 48 h;  $n = 24$  per tissue). Hydrolysis rate of an equimolar emulsion of tri-C6:0 and tri-C8:0 (200 mM each) was assayed. Activity was expressed as micromoles of fatty acid liberated per minute per total organ weight (means  $\pm$  SEM).

rat pancreatic amylase, follows this time course, and may be a common feature of the digestive enzymes (Hamosh and Hand, 1978). Low preduodenal lipase activity apparently is common to the rabbit also. Fetal rabbits 15 to 30 d into gestation had little or no gastric lipase activity; however, by 6 d of postnatal age significant activity could be detected (Bernadac et al., 1991). The rapid increase in gastric lipase activity during the suckling period reached a peak at 6 wk of age, followed by a marked decrease in activity after weaning.

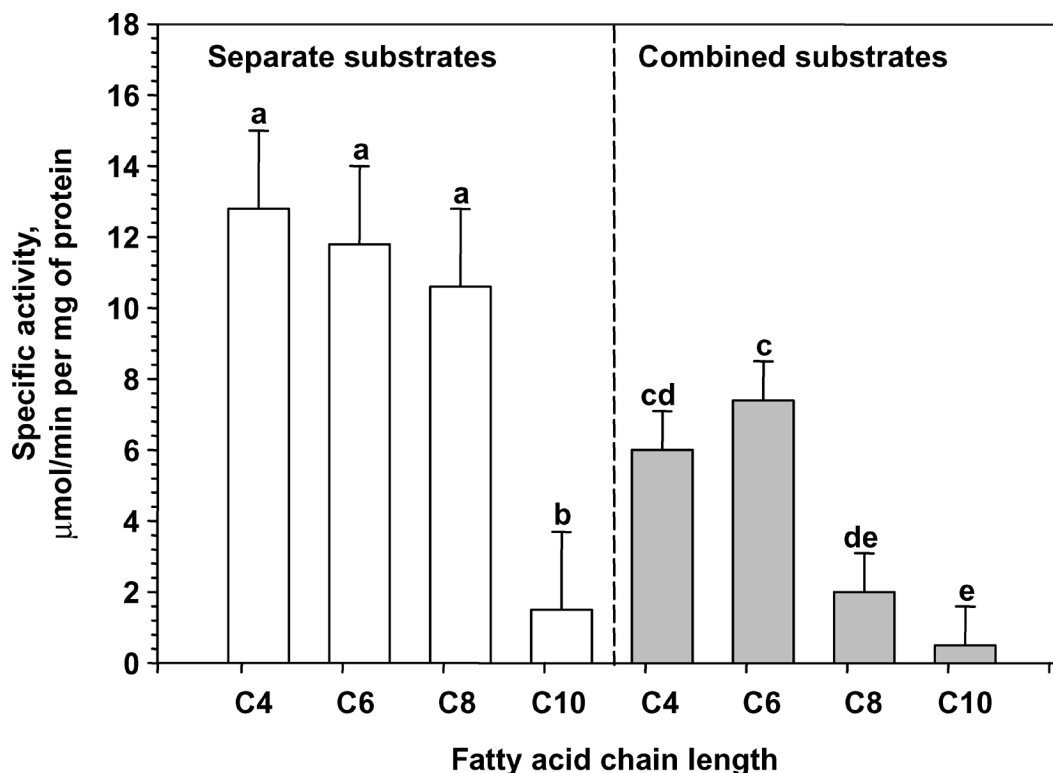
#### Chain-Length Specificity

Congruent with our previous *in vivo* studies (Wieland et al., 1993a; Odle et al., 1994), tri-C6:0 was digested and absorbed more rapidly than tri-C8:0, as indicated by 7.5-fold greater plasma free fatty acid concentration 1 h after intubation. This dramatic effect of chain-length (6 vs. 8 carbons) could be explained in part by preferential hydrolysis of tri6:0 by pancreatic lipase.

The chain-length specificity of pancreatic lipase was determined initially from a mixed lecithin emulsion of tri-C6:0 and tri-C8:0 substrate assayed at 0, 6, 18, and 48 h postnatally. Hydrolysis of tri-C6:0 was 5-fold

greater than that of tri-C8:0 ( $P < 0.001$ ). Further examination of chain-length specificity utilized 2 different assays. In the first, the substrates (tri-C4:0, tri-C6:0, tri-C8:0, and tri-C10:0) were combined, and their hydrolysis measured simultaneously. In the second, the substrates were incubated with lipase separately and their hydrolysis measured individually. In both cases, pancreatic tissue from the same 3 piglets at 6 h of age were assayed using substrates emulsified with lecithin and present in equimolar amounts. When substrates were measured separately, the activities appeared to decrease progressively as chain length increased. When substrates were combined, tri-C6:0 was hydrolyzed the fastest. In both instances, tri-C10:0 was lower ( $P < 0.05$ ), with activity toward tri-C10:0 representing about 10% of the activity toward tri-C6:0.

Most studies regarding chain-length specificity of pancreatic lipase compare short-chain with long-chain fatty acids. According to published literature (Erlanson and Borgstrom, 1970; Lairon et al., 1980; Gargouri et al., 1986), tri-C4:0 is hydrolyzed twice as fast as tri-C18:0. More relevant are the hydrolysis rates of porcine pancreatic lipase reported by Desnuelle and Savary (1963). Congruent with respective water solubilities,



**Figure 5.** Chain-length specificity of pancreatic lipase in neonatal piglets. Hydrolysis rates were measured both on a triacylglycerol mixture containing equimolar tri-C4:0, tri-C6:0, tri-C8:0, and tri-C10:0 (solid bars; 400 mM total) and on each triacylglycerol substrate assayed separately (open bars; 400 mM). Lipase activity was measured in 6-h-old piglets, and expressed as micromoles of fatty acid liberated per minute per milligram of protein. <sup>a-e</sup>Within an assay, means  $\pm$  SEM (n = 3) lacking a common superscript letter are different ( $P < 0.05$ ).

they determined tri-C4:0 to have the greatest rate of hydrolysis, with longer chain lengths being hydrolyzed at steadily decreasing rates until tri-C10:0. Triacylglycerols with longer chain lengths than C10:0 were hydrolyzed at very similar rates, well below those of medium-chain triacylglycerols. These results correlate strongly with the findings from our experiments, when the substrates were measured separately. Accordingly, tri-C4:0, tri-C6:0, and tri-C8:0 all appear to be good substrates for pancreatic lipase. However, when substrates were combined, tri-C8:0 was hydrolyzed at a diminished rate. Physical and kinetic properties of C8:0 place it at a substantial disadvantage when in combination with triacylglycerols of smaller chain length. Both tri-C6:0 and tri-C4:0 may predominate at the oil-water interface of the lipid emulsion, compared with tri-C8:0 and tri-C10:0, allowing for preferential hydrolysis of C6:0 and C4:0. Thus, the greater hydrolysis could be attributed to the specific physicochemical properties of tri-C6:0 and tri-C4:0 rather than chain-length specificity of the enzyme per se. If there were a true specificity of pancreatic lipase for tri-C6:0 compared with tri-C8:0, differences would have been observed when measuring activity on each substrate separately. Possibly the only substrate reflective of chain-length specificity was tri-C10:0. In all instances, it was hydrolyzed more slowly than the other triacylglycerols. As was observed for

pancreatic lipase, the hydrolysis of tri-C6:0 by gastric and lingual lipase was greater than that of tri-C8:0 by a factor of 7. It should also be noted that medium-chain triglycerides might be hydrolyzed by lipases located within intestinal enterocytes (see Bach and Babayan, 1982). Although we did not attempt to measure this intestinal source of lipase, it is possible that it contributes importantly to MCT hydrolysis, and may be responsible in part for the chain-length effects observed.

Positional stereospecificity was not examined for any of the lipases. The MCT used were all synthetically derived to contain only a single fatty acid chain length. Therefore, the differences in chain-length hydrolysis could not be attributed to preferential hydrolysis at the sn-3 position (Jensen et al., 1982; Rogalska et al., 1990). Often, naturally occurring triacylglycerols have the medium-chain fatty acid moiety located at the sn-3 position (Smith and Abraham, 1975), explaining the greater rate of medium-chain fatty acid release compared with longer chain-length fatty acids, which are commonly at the sn-2 or sn-1 position.

In summary, the results from this study confirm the observation that medium-chain triglycerides containing hexanoate are utilized more rapidly by the newborn piglet than those containing octanoate. However, most commercial preparations of medium-chain triglycerides are composed of octanoate and decanoate. This

study further demonstrates that the accelerated kinetics of hexanoate use are due in part to greater pancreatic lipase activity toward trihexanoate substrate, and that lingual and gastric lipase contribute relatively little to the overall lipase activity in the newborn piglet.

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