Activities of gastric, pancreatic, and intestinal brush-border membrane enzymes during postnatal development of dogs

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Objective—To measure activities of digestive enzymes during postnatal development in dogs.

Sample Population—Gastrointestinal tract tissues obtained from 110 Beagles ranging from neonatal to adult dogs.

Procedure—Pepsin and lipase activities were measured in gastric contents, and amylase, lipase, trypsin, and chymotrypsin activities were measured in small intestinal contents and pancreatic tissue. Activities of lactase, sucrase, 4 peptidases, and enteropeptidase were assayed in samples of mucosa obtained from 3 regions of the small intestine.

Results—Gastric pH was low at all ages. Pepsin was not detected until day 21, and activity increased between day 63 and adulthood. Activities of amylase and lipase in contents of the small intestine and pancreatic tissue were lower during suckling than after weaning. Activities of trypsin and chymotrypsin did not vary among ages for luminal contents, whereas activities associated with pancreatic tissue decreased between birth and adulthood for trypsin but increased for chymotrypsin. Lactase and γ-glutamyltranspeptidase activities were highest at birth, whereas the activities of sucrase and the 4 peptidases increased after birth. Enteropeptidase was detected only in the proximal region of the small intestine at all ages.

Conclusions and Clinical Relevance—Secretions in the gastrointestinal tract proximal to the duodenum, enzymes in milk, and other digestive mechanisms compensate for low luminal activities of pancreatic enzymes during the perinatal period. Postnatal changes in digestive secretions influence nutrient availability, concentrations of signaling molecules, and activity of antimicrobial compounds that inhibit pathogens. Matching sources of nutrients to digestive abilities will improve the health of dogs during development. (Am J Vet Res 2003;64:627–634)

Before proteins, fats, and carbohydrates in feedstuffs can be absorbed, they must be hydrolyzed by a sequential process that yields the constituent amino acids and small peptides, fatty acids and monoglycerides, and monosaccharides. This is accomplished by enzymes secreted into the lumen of the gastrointestinal tract (GIT) and by another set of enzymes in the brush-border membrane (BBM) of enterocytes.

Incomplete hydrolysis can limit the availability of energy and nutrients in feedstuffs. Therefore, postnatal changes in digestive secretions, including the activities and relative proportions of the various luminal and BBM digestive enzymes, can be predicted to influence the digestion of dietary inputs and availability of nutrients. The best example is the reciprocal shift in the activities of lactase and sucrase-isomaltase at the time of weaning and the ability of developing mammals to tolerate lactose and sucrose, respectively. Although the pancreas develops before birth, it is functionally immature at the time of birth. Corresponding to this, the activities of pancreatic enzymes are low during early postnatal development, and most newborn mammals are characterized as having pancreatic insufficiency. Mature functions are not attained until later, generally at or after the time of weaning. Similarly, activities of BBM peptidases are lower during suckling, compared with activities after weaning. Consequently, limited digestive capacities of dogs during suckling may limit nutrient availability; such limitations have been speculated for cats.

Patterns of development for the exocrine pancreas and the BBM are species-specific and set by genetic determinants with some modulation by dietary inputs and hormones. Consequently, the findings for 1 species or diet regimen may not be applicable for understanding development of the GIT in another species or for other conditions.

Dogs have been used extensively for biomedical research of digestive processes. Responses of the mature digestive tract (including digestive secretions) to secretagogues or other stimuli and to diets with alternative sources of nutrients have been evaluated. However, there is little known about the postnatal changes in digestive secretions of dogs, despite the obvious relevance to nutrition and health. The objective of the study reported here was to describe changes in the activities of enzymes associated with the hydrolysis of proteins, carbohydrates, and fats in Beagles during development. The study involved assaying the activity of enzymes in contents of the stomach and small intestine, in pancreatic tissue, and associated with the BBM of the small intestine. Results of other studies in which these dogs were used to examine characteristics of the small intestine during development have been reported elsewhere.
Materials and Methods

Animals—Ninety-five puppies representing 15 litters and the 15 dams of those litters were used to conduct the study. Dogs were procured, housed, and fed as described elsewhere.12 Digestive secretions and BBM enzymes were measured in tissues obtained from 6 groups of dogs (unsuckled neonates within 1 hour after birth [n = 14]; 1-day-old neonates after initial suckling [20]; puppies at 21 [19], 42 [21], and 63 [21] days of age; and adult females [ie, dams of the puppies; 15]).

Collection of samples—Dogs in each group were euthanatized. Within 5 minutes after each dog was euthanized, contents of the stomach and mid region of the small intestine were collected into vials and immediately placed on ice. Mucosa was scraped from the proximal, mid, and distal regions of the small intestine (the proximal region represented the proximal portion of the jejunum, mid region represented the distal portion of the jejunum, and distal region represented the ileum), rapidly frozen, and stored at –80°C. The pancreas was removed, weighed, and stored at –80°C.

Measurement of luminal enzymes—Luminal contents of the stomach and small intestine were centrifuged (4,000 × g for 5 minutes). The pH of the supernatants was recorded, and supernatants were retained on ice for immediate assay of enzyme activities.

Activity of pepsin (EC 3.4.23.1) in gastric contents was measured by use of denatured hemoglobin as the substrate.13 Lipase (EC 3.1.1.3) activity in the gastric and small intestinal contents was measured colorimetrically by use of a diagnostic kit14 in which 4,6-ethylene (G 7)–glutamyltransferase activity was obtained by incubating the supernatant with 8.0 units of enteropeptidase at room temperature (pH, 7.4) with 0.02mM CaCl2 (0.025 to 0.05 g of pancreatic tissue was homogenized in 0.05mM Tris buffer [pH, 6.4], with intermediate values for the other ages. We were not able to measure pH in 1-day-old puppies because of the small volume of intestinal contents at that time.

Measurement of enzymes in pancreatic tissue—Frozen pancreatic tissue was homogenized in 0.05mM Tris buffer (pH, 7.4) with 0.02mM CaCl2 (0.025 to 0.05 g of pancreas/ml). Homogenates were centrifuged twice at 4°C (2,420 × g for 5 minutes, then 16,000 × g for 5 minutes). After the second centrifugation, the supernatant was divided into aliquots and stored at –80°C. Supernatants were diluted (same buffer used for tissue homogenization) so that measured activities of each enzyme would be within the dynamic range of the assay and were comparable to standards with known activities prepared by use of trypsin,15 chymotrypsin,16 amylase,4 and lipase.7 Activities of trypsin, chymotrypsin, and lipase were assayed as described previously. Enteropeptidase was used to activate trypsinogen and chymotrypsinogen, which are found in pancreatic tissue as inactive zymogens. Preliminary studies revealed that maximum trypsin and chymotrypsin activity was obtained by incubating the supernatant with 8.0 units of enteropeptidase at room temperature (20 to 22°C) for 1 hour and then measuring trypsin and chymotrypsin activities. Amylase activity of the pancreatic tissue homogenates was measured colorimetrically by use of a diagnostic kit17 in which 4,6-ethylene (G 7)–glu-nitroanilide release was measured at 410 nm by use of specific substrates to assay for the activities of aminopeptidase A (EC 3.4.11.7),18 aminopeptidase N (EC 3.4.11.2),19 γ-glutamyltranspeptidase (GGT; [EC 2.3.2.2]),20 dipeptidyl-dipeptidase IV (EC 3.4.14.5),21 and enteropeptidase (EC 3.4.21.9).22 Activities of the enzymes were adjusted on the basis of protein content (specific activity) by use of a protein assay reagent23 with bovine serum albumin as the standard.

Analysis of data—Values were reported as mean ± SEM. Age effects were detected by use of an ANOVA with specific differences identified by use of the Duncan test. For all comparisons, values of P ≤ 0.05 were accepted as significant.

Results

Gastric contents—Pepsin activity was not detected in gastric contents obtained from 1-day-old puppies. The pH in gastric contents of 1-day-old puppies (3.0) did not differ significantly from values recorded in gastric contents of older dogs, which ranged from 2.5 in adult dogs to 3.9 in 63-day-old puppies. Mean ± SEM pepsin activity did not differ between gastric contents obtained from puppies at day 1 (126 ± 28 U/g of gastric contents), day 21 (42 (601 ± 248 U/g of gastric contents), and day 63 (475 ± 202 U/g of gastric contents) days of age, but values in gastric contents of adult dogs (2,208 ± 524 U/g of gastric contents) were significantly higher than for gastric contents obtained from the puppies. Lipase activity was detected in the gastric contents obtained from dogs of all ages, but values for suckling dogs (day 1, 10.3 U/g of gastric contents; day 21, 107 U/g of gastric contents; day 42, 48 U/g of gastric contents) were significantly higher than those for 63-day-old puppies (0.29 U/g of gastric contents) or adult dogs (0.31 U/g of gastric contents).

Contents of the small intestine and pancreatic enzymes—The pH in the small intestinal contents differed significantly among the groups. It was lowest in 42-day-old puppies (pH, 5.4) and highest in adult dogs (pH, 6.4), with intermediate values for the other ages. Protein content of pancreatic tissues decreased significantly from 39 ± 2 mg/g at day 1 to 30 ± 1 mg/g at day 42. Values increased thereafter and were significantly higher in adult dogs (53 ± 3 mg/g), compared with values for all other ages.

Trypsin activity of pancreatic tissue was highest during suckling (days 1 to 42), with the lowest values measured at day 63 (Fig 1). In contrast, trypsin activity in the luminal contents was significantly lower on day 1 but did not differ significantly among the other ages. Chymotrypsin activity of pancreatic tissue increased between days 1 and 21, with another increase between day 63 and the adult dogs. Specific activity for chymotrypsin in the luminal contents was significantly higher at day 63, compared with activity at day 1, but had similar values for all other ages.
Amylase activity was not detected in the pancreatic homogenates or luminal contents at day 1 (Fig 1). Low values were first detected in pancreatic tissue and luminal contents at day 21. The activity in the pancreatic tissue increased significantly (>10-fold) between day 63 and the adult dogs but decreased slightly in the luminal contents during the same period.

Lipase activity was not detected in pancreatic homogenates for puppies ≤42 days of age, but high amounts of activity were detected for puppies at day 63 and adult dogs (Fig 1). Low amounts of lipase activity were detected in intestinal contents of suckling puppies. Activity in the luminal contents peaked in 63-day-old puppies, with a decrease in measured activity in adult dogs.

An additional indicator of age-related changes in the pancreatic enzymes was determined. Ratios were calculated for the activities of amylase, lipase, and chymotrypsin relative to trypsin. Amylase-to-trypsin ratios for pancreatic tissue increased significantly (P < 0.001) from 0 at day 1 to 0.09 ± 0.01 in the adult dogs. A similar age-related significant (P < 0.001) increase was
detected for lipase-to-trypsin ratios, but the increase was after day 42, with the highest ratios at day 63 (0.027 ± 0.002). Chymotrypsin-to-trypsin ratios were similar from days 1 to 42, then increased significantly for 63-day-old puppies and adult dogs.

**BBM enzymes**—Mean values for the enrichment factors for the BBM enzymes were > 5 for the various ages and 3 regions of the small intestine. These findings indicated that the BBM had been isolated from the homogenate. However, mean recovery values were < 20%, suggesting that only a portion of the BBM was isolated or, alternatively, that a substantial fraction of enzyme activity was intracellular and not associated with the apical membrane.

Lactase activity was detected in homogenates (data not shown) and BBMV from days 0 to 21 but not thereafter (Fig 2). Sucrase activity was first detected at day 21, but this activity did not increase significantly until between day 63 and adulthood (Fig 2). When lactase and sucrase activities were detected, activities decreased from the proximal to the distal region of the small intestine, with the exception of lactase at 21 days.

Two patterns of distribution were evident in the small intestine for the activities of the 4 BBM peptidases (ie, aminopeptidases A and N, LAP, and dipeptidyl-dipeptidase IV; Fig 3). A proximal-to-distal gradient was evident in dogs ≥ 21 days old for aminopeptidase N and LAP but not for aminopeptidase A and dipeptidyl-dipeptidase IV. When all 3 regions were pooled, the activities of aminopeptidases A and N and dipeptidyl-dipeptidase IV were lower at day 0 with a gradual increase into adulthood. The activity of LAP was lowest at day 0 but similar at all other ages.

The activity of GGT did not vary among age groups. It also did not have a consistent pattern of distribution among the 3 regions of the small intestine (Fig 4). We did not detect Na⁺-K⁺-ATPase in BBMV and homogenates of puppies at day 0 (data not shown), and there was only low activity detected in BBMV and homogenates at day 1. The highest activities were detected at 21 days with lower and similar activities for puppies at days 42 and 63 and the adult dogs.

Enteropeptidase activity was detected in BBMV and homogenates at all ages; however, it was detected only in the proximal region of the small intestine. Activity was highest at days 0 and 1, compared with activity for all other ages (data not shown).

**Discussion**

To our knowledge, the study reported here is the first in which changes in digestive enzyme activities of dogs have been measured from birth to adulthood. Although only a limited number of enzymes were evaluated, the lower activities of some pancreatic and BBM enzymes revealed the fact that hydrolytic capacities of suckling puppies differ markedly from those of weaned and adult dogs. Lower hydrolytic capacities have led to the contention that neonatal animals have pancreatic insufficiency despite having a fully formed pancreas at birth. This speculation is corroborated by the decrease in protein content of the pancreas during early suckling that was reported here, which suggests enzyme
development and functions. Consequently, neonatal colostrum that may be important for regulating GIT activity provided additional evidence that luminal activities are paralleled, as evident by the greater proportional increase after weaning for amylase and lipase compared to trypsin. In addition, maturation of digestive secretions was not complete by week 9 based on comparisons with the adult dogs. Although unknown for Beagles, the lack of differences between 12 and 20 weeks for the digestibility and responses of Labrador dogs to different diets suggests GIT digestive processes for that breed mature between weeks 9 and 12.

Lack of pepsin activity in the gastric contents at day 1 is consistent with the limited secretion of gastric proteases reported for other suckling mammals, including humans. These findings, in conjunction with the low activities of trypsin in luminal contents, suggest that newborn dogs have only limited abilities to hydrolyze dietary protein. The combination of low pH of the stomach contents of the dogs reported here, high protease activities in the gastric mucosa of neonatal dogs and pigs, high trypsin activity in pancreatic tissue, and enteropeptidase activity in the proximal region of the small intestine of the dogs of this study indicate that low luminal proteolytic activity during the perinatal period is limited by secretion, not by synthesis or activation. Similarly, peptidase activity associated with the BBM was lower for neonatal puppies, compared with activity for older puppies or adult dogs. Because activity was consistently detected in homogenates at all ages, insertion into the apical membrane, and not a decrease in synthesis, was the probable cause of the low BBM activity. Chymotrypsin activity provided a contrasting pattern, with low activity in luminal contents and pancreatic tissues throughout all ages. Despite the age-related increase in chymotrypsin activity, dogs are more dependent on trypsin for luminal digestion of dietary proteins. Interestingly, the chymotrypsin-to-trypsin ratio in pancreatic tissue increased nearly 7-fold after weaning, but an age effect was not detected for the luminal contents. This provides additional evidence that luminal activities are regulated more by secretion than by synthesis.

The combination of low protease activity in the luminal contents and BBM during the neonatal period and protease inhibitors in milk (particularly colostrum) appears to be adaptive. It has been speculated that limited protease activity allows for the acquisition of passive immunity and preserves the high concentrations of signaling molecules contained in colostrum that may be important for regulating GIT development and functions. Consequently, neonatal dogs may be reliant on endocytosis by the small intestine and intracellular digestion of protein. Although the information is not known for dogs, other species have postnatal decreases in endocytosis and a shift from intracellular digestion to an increasing reliance on luminal hydrolysis.

Postnatal changes in protease activities vary among species and enzymes. For example, there are several forms of gastric proteases that have differing hydrolytic characteristics. In the late-fetal and early-suckling periods of development, the stomach synthesizes and secretes chymosin, which is important for clotting milk but has weaker hydrolytic properties compared to the pepsin isoforms secreted by the mature stomach. This may explain the reduced ability of gastric contents from neonatal dogs to hydrolyze hemoglobin. Conversely, pepsin secreted by the gastric mucosa of mature dogs is less able to clot milk. Apparently, secretion of the pepsin isoforms and other gastric aspartic proteases that are characteristic of mature dogs increases during suckling, with adult values achieved sometime after weaning. A similar pattern of postnatal development for the activities and types of gastric proteases has been reported for cats.

Low lipase activity in the pancreatic tissue and luminal contents of the small intestine during early postnatal development of dogs and ferrets, in conjunction with immature bile secretion and undeveloped intestinal absorption and recycling of bile acids, is not consistent with the high fat content in the milk of bitches. Instead, neonatal and suckling puppies have a greater dependency on lipase activity in the GIT proximal to the duodenum. This includes 3 sets of enzymes that originate from nonpancreatic tissues, each of which have unique characteristics and distinct patterns of development.

Milk collected from dogs has lipase activities that are similar to those measured in human milk but that exceed those in the milk of cats. Colostrum has lower lipase activity, compared with the activity for milk, and this is consistent with the lower activity measured in the contents of the stomach and small intestine at day 1, compared with activity measured at day 21. Lipase is also secreted by the gastric mucosa of suckling dogs and cats, and to 30% of milk fat is hydrolyzed in the stomach of suckling rats. Gastric lipases can be differentiated from those secreted by the pancreas on the basis of stereospecificity and optimal pH. The reciprocal decrease in gastric lipase activity and increase in pancreatic lipase activity at the time of weaning coincided with the loss of milk lipase activity, which indicates that there is a shift in the relative importance of the various sources of lipase. Although lingual lipase is secreted by suckling rats, the potential contribution to lipid digestion is unknown for suckling puppies and is minimal or lacking for adult dogs. The shift to a greater dependency on pancreatic lipase between the suckling period and adulthood was evident by the 1,000-fold increase for the lipase-to-trypsin ratio in the luminal contents of the small intestine.

The source of fat and digestion of fat have an interesting relationship that can have other important roles in early development. Specifically, hydrolysis of
milk fat, but not other lipids, inhibits parasites. \(^{38,39}\) Furthermore, digestion of fat and the release of fatty acids (e.g., oleic acid) stimulates secretion of cholecystokinin and other regulatory peptides \(^{40-42}\) that have trophic influences on the mucosa. \(^{61}\) It is unknown whether feeding suckling puppies milk replacers or solid diets with alternative sources of lipids would reduce pancreatic secretion, alter development of the GIT, or influence health of the developing dogs.

The milk of bitches also contains amylase \(^{64,65}\) and ribonuclease \(^{66}\) activity. This explains the reason that low but measurable amounts of amylase activity were detected in the contents of the small intestine at days 1 and 21, despite the virtual lack of amylase activity in pancreatic tissue at day 1 and the negligible amount of activity at day 21. The increase in amylase activity in pancreatic tissue and luminal contents of the small intestine after day 21 coincided with the period when the puppies began to eat the weaning diet, which contained starch and other complex carbohydrates. Another increase in amylase activity in pancreatic tissue was detected when the dogs were weaned (day 63) and in the adult dogs \(^{30}\); the maintenance diet for the adult dogs had a higher proportion of carbohydrate than did the weaning diet. However, this did not result in higher amylolytic activity in luminal contents of the small intestine. This further documents that secretion, not synthesis, limits activity in the luminal contents.

The role of GGT in cystine absorption and glutathione metabolism is critical for suckling Beagles \(^{67}\) and for function of the GIT. \(^{68-70}\) Activity has been detected in numerous tissues, \(^{71}\) including the small intestine of dogs, \(^{2}\) and the higher activities in sucking puppies, compared with activities for weaned and adult dogs reported here, is similar to findings \(^{72,74}\) for rodents and other species.

Although Na\(^+\)-K\(^+\)-ATPase activity was detected in the BBMV, the majority of activity is associated with the basolateral membrane of enterocytes and colonocytes. \(^{73,76}\) The low amounts of Na\(^+\)-K\(^+\)-ATPase activity in the small intestine of neonatal dogs reported here and the colon of infant rats reported in another study \(^{72}\) suggest the mechanism of potassium absorption changes after the neonatal period. The postnatal peak in activity agrees with findings for rodents and with the higher rates of absorption and potassium requirements for suckling animals, compared with values for adult animals. \(^{73,77}\)

In some animals, patterns of development for the digestive enzymes reflect a combination of genetic determinants interacting with dietary inputs. \(^{1}\) The increase in amylase activity after day 21 is consistent with starch in the weaning diet but not in milk. This suggests that dietary inputs play an important role. In contrast, there was a loss of lactase activity in the BBM and homogenate between days 21 and 42, even though the dogs continued to consume milk, which has a relatively constant lactose content. \(^{16}\) In addition, sucrase activity was initially detected at 21 days, even though sucrose is not found in milk and the puppies had not yet started to eat the solid weaning diet. These findings indicate that genetic determinants, not diet, trigger reciprocal changes in the activities of lactase and sucrase. Interestingly, specific activity of lactase increased during the first 21 days after birth, whereas rates of aldo-sucrase transport decreased. \(^{41}\) These findings indicate that the activities of BBM enzymes and the associated transport functions may not be matched, which is the situation seen in older animals. \(^{72,79}\)

The relative importance of genetic determinants and dietary inputs in mediating the changes in protease activities are less obvious, because the protein content of the weaning diet was not higher than that of milk of the dams. Instead, the digestibility of proteins in the plant materials used to formulate diets for dogs is lower\(^{6}\) and more variable, \(^{7}\) compared with digestibility of animal tissues, and this may trigger a compensatory increase in the synthesis and secretion of pancreatic proteases. It is unlikely that the increase was stimulated by protease inhibitors associated with the plant ingredients. \(^{80}\)

Weaning diets are prepared by use of a combination of animal tissues and plant materials. However, the age-related changes in digestive secretions may limit the ability of young dogs to digest some of the components. A better understanding of the patterns of development for digestive secretions, associated mechanisms, and signals triggering the changes will assist in the formulation of diets that are matched to the digestive capacities of specific stages of development in dogs.

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References

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