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ULTRASTRUCTURAL STUDIES OF MILK DIGESTION IN THE SUCKLING RAT

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Abstract

The structure of milk in the stomach and proximal small intestine of suckling rats at 12 and 24 hours, 5, 10 and 15 days of age was examined by scanning and by transmission electron microscopy. The milk curd in the gastric lumen consisted of chains of casein micelles which entrapped milk fat globules. The appearance was similar to that reported for bovine milk curd and cottage cheese. The gastric milk curd at 12 and 24 hours also contained large masses of granules 4-8 nm in diameter. The relationship of these particles to casein micelles is unknown.

Casein micelles in the duodenum appeared to disperse irrespective of age. In the central duodenal lumen milk fat globules lost their encircling membranes and underwent lipolysis, evidenced by progressive peripheral irregularity as they passed between the lateral regions of the villi.

Introduction

Milk is the sole source of nutrition for newborn humans and other mammals during the suckling period. Milk and milk products are also of great dietary and economic importance as food components. The main particulate components of milk are fat globules and casein micelles. Triacylglycerol in milk fat provides a major portion of the caloric content in milk. The processes of the synthesis of milk by cells in the mammary glands of several species have been reviewed by Linzell and Peaker (27). Casein micelles consist primarily of \( \alpha_s \)-casein, \( \beta \)-casein and \( \kappa \)-casein (41) in bovine milk. The location of the \( \kappa \)-casein component in bovine milk casein has been of much interest (10,26,31,33,34). Possible similarities in the structure of bovine and rat casein particles are not yet clear.

Whereas rat mammary tissue has frequently been used as a subject for the examination of the synthesis of milk proteins and fats (12,35) structural changes that occur following the ingestion of milk by suckling animals have received little attention.

Digestive enzyme levels and biochemical specificities have been reported to differ significantly when those of suckling rats are compared to those of adult rats. In the oral cavity amylase of parotid origin is at low levels of activity until weaning. Lingual lipase activity levels are high but not effective within the oral cavity. Pepsin activity is lacking in the stomach until weaning. In the small intestine chymotrypsin is active at birth but trypsin, pancreatic lipase and amylase are at low activity levels until about day 15 of suckling (19). During the suckling period ingested milk is exposed to these regional enzymes. The changes in appearance of milk fat globules and casein in the stomach and small intestine of suckling rats of different ages are the subjects of this report.

Materials and Methods

Pregnant Sprague-Dawley rats were received "due to deliver in 3-4 days" and were watched so that the dates of birth of the rat pups were accurately recorded. The rats were housed in a controlled environment and had constant access...
to food and water. In a few experiments lactating rats were fed ground lab chow which contained corn oil (15% by weight) to enhance the unsaturated fatty acid content of the milk.

The rat pups were decapitated and short pieces of small intestine from within 4 cm of the pylorus and fragments of the gastric milk curd layers were taken for electron microscopy.

The specimens were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 for 30 minutes at 4°C. For transmission electron microscopy the fixed specimens were then washed in 0.1 M cacodylate buffer pH 7.3 with 10% sucrose and were post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.3 for 2 hours. Some specimens were fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.3 for 2 hours.

The specimens were dehydrated in a series of cold acetones over a 65-minute period, brought to room temperature in the final acetone, passed through acetone-epon mixtures and were embedded in epon. Some specimens of intestine were dehydrated in Durecupan Fluka and embedded in a mixture of Durecupan and Araldite. One-micron-thick sections were examined for orientation and the appropriate areas were selected for thin sectioning. Sections were cut on Reichert OMU-2 or LKB ultramicrotomes, picked up on 200 or 75 x 300 mesh grids, stained with uranyl acetate and lead citrate and examined with a Philips EM-300 electron microscope (3,4).

Specimens for scanning electron microscopy were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 for 24 hours, were washed in 0.1 M cacodylate buffer pH 7.3 with 10% sucrose and were post-fixed for 2 hours in 2% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.3. After again washing in buffer the specimens were dehydrated in ethanol and were critical point dried. The mounted specimens were sputter coated to produce an approximately 20 nm thick layer of gold. The specimens were examined with an AMR 1200 B scanning electron microscope.

Observations and Discussion

Casein in Gastric contents
Newborn rats suckle most of the daytime and have continually full stomachs. This gastric curd consists of concentric solid layers separated by liquid strata of whey. Each solid layer is a mixture of casein micelles and fat globules as recognized by Platt (32). Ingested milk curd may remain in the stomach as long as 20 hours and
Fig. 5. Milk fat globules (F) are surrounded by casein particles. A fragment of cytoplasm, including casein particles is at arrow. TEM

Fig. 6. Sectioned casein micelles in gastric curd have a patterned opacity. TEM

Fig. 7. A casein micelle in the gastric curd has parallel rows of electron-opaque substance (arrows). TEM Photo, courtesy reference 4.

Fig. 8. Homogeneous masses of milk protein (P) are present within the gastric curd at 24 hours of age. Phase contrast of Epon sections.

there appears to be a continued release of softened curd at the pyloric antrum which provides to the duodenum a continual flow of milk components in various stages of digestion (32).

Light microscopy of gastric milk curd from rats 12 and 24 hours, 5, 10 and 15 days old, showed irregularly arranged fibers about 1 μm wide which surrounded fat droplets (Figure 1).

Scanning electron microscopy (SEM) showed rounded or oval fat globules approximately 1-10 μm in diameter in an irregular mass (Figure 2). Higher magnifications showed chains of 0.1 μm particles on the surface of the fat globules (Figures 3 and 4). These 0.1 μm particles were casein micelles. The appearance of the casein curd and chains of micelles was similar to that of milk gels produced by heat and acid (23) and also similar to that of acid set curd of cottage cheese (13,14,24).

Transmission electron microscopy (TEM) showed the sectioned fibers to be composed of clumps and chains of oval casein particles. These had diameters of approximately 0.1 μm and appeared to be fused at the points of contact (Figure 5).

The sectioned particles were composed of electron opaque and less opaque areas. In most casein particles the dense areas appeared as irregularly interconnected polygonal rings (Figure 6). In some sectioned particles, the opaque areas were organized in parallel lines at intervals of approximately 20 nm (Figure 7) as previously reported (4).

Chemical analyses of the fractions of rat casein micelles are presently of much interest. Recent reports (20,36,40,41) have identified at least three major components with one, m.w. 25,000, being homologous to bovine β-casein.

The coagulative linking of these casein particles has probably resulted from a change in pH. Koldovsky (25) has reported that the gastric lumen of newborn rats is pH 5.6-6.6. Parietal cells are present at birth and gastric acid production is reported to be stimulated by the first ingestion of milk (29). The gastric pH drops to 3.0-4.6 during the first two days of life (25). Woodward and Messer (40) reported that rat casein precipitates at pH 4.0-5.0. Slattery has suggested that the τ-casein component in bovine
Fig. 9. Homogeneous masses of milk protein are fine particles as seen with the transmission electron microscope.

Fig. 10. The particles in Figures 8 and 9 vary in opacity and in size at high magnification in the transmission electron micrograph.

Fig. 11. Casein particles (arrow) in the duodenal lumen at 10 days of age lost their irregular pattern of opacity. TEM

Fig. 12. Milk fat globules in the central duodenal lumen of a 10 day old rat have ruffled surfaces (arrow). SEM

Milk micelles may be the anchor points for formation of the chains of the coagulum (34). Bovine caseins have been shown to be digested in the stomach of the adult rat (28). In the stomachs of suckling rats, casein micelles in the chains of the coagulum did not appear to be dispersing. Whereas parietal cells have been shown to produce acid at birth (29), pepsin is not produced until after weaning at about day 25, and therefore proteolytic activity of the gastric contents is probably low (25).

Gastric contents of 12-and 24-hour old suckling rats included large lobulose homogeneous masses. Phase microscopy showed these sectioned masses as light gray and surrounded by fat droplets. They were adjacent to masses of casein micelles (Figure 8). By TEM, they were shown to be smooth bordered and appeared as tightly packed confluent masses of 4-8 nm diameter particles (Figure 9). Individual particles differed in electron opacity (Figure 10). Vacancies with varying diameters were present within the compact masses. Some of these were devoid of opaque particulate matter while others contained free granules, small clumps of granules, membrane fragments or lipid droplets. These masses of fine particles have not been reported by other investigators. Perhaps they are a form of casein in colostrum (4).

Milk Fat Globules in Gastric Contents

By light microscopy, the milk fat globules in the curd fragments were found to be surrounded by casein particles (Figure 1). In the SEM, the disrupted curd showed chains of small oval particles which covered the fat globules in a net-like fashion (Figures 3 and 4). The TEM revealed that the fat globules were round and were smooth in outline, and had homogeneously electron opaque cores incompletely surrounded by single or multilaminar membrane fragments (3). Fat globules of varying opacity were near the periphery of the gastric milk curd. Membranes encasing milk fat globules have been noted in the breast milk of several species as reported by Wooding et al. (37,38,39) and by Linzell and Peaker (27).
Lipolytic activity within the stomach has been identified by several investigators (16,18). Recent identification (15,16) of a strong lipase produced by the von Ebner glands of the tongue may explain gastrointestinal lipolysis since enzymes hydrolyze medium and long chain fatty acids. Ester bonds of medium chain fatty acids and long chain unsaturated fatty acids are hydrolyzed at higher rates than those of long chain saturated fatty acids (1,16,21). No structural signs of lipolysis were observed in the gastric contents in this study. Perhaps lipolysis is occurring in the soft portion between the layers of gastric curd and is therefore lost in preparation of curd fragments. My specimens were prepared from the centers of gastric curd layers and would be expected to miss superficial fat globules. Gastric epithelial cells of suckling rats have been found to absorb medium-chain length fatty acids but the significance is not known (9).

Fat globules with associated cytoplasmic remnants were occasionally present as described by Wooding et al. (39) and by Linzell and Peaker (27). These cytoplasmic fragments have originated from loss of portions of the mammary epithelial cells at the time of fat droplet extrusion and can usually be identified by the presence of casein micelles within. Vegetable fragments with bacteria adjacent were also seen in the stomach contents. These may have originated from accidentally ingested bedding material.

Casein in Duodenal Contents

During suckling, the yellow-white color of wall of the proximal and jejunal portions of the rat small intestine is probably a result of the intraluminal fat being absorbed. The distal (ileal) portion has a yellow-brown color. Most of the casein particles in the duodenal lumen of suckling rats were not adherent to one another. As shown by TEM, those which were still linked had a pattern of different opacities, but appeared as clumps of fine particles (Figure 11). Many micelles appeared to be fragmenting. The homogeneous masses noted in the gastric content at 24 hours of age did not have smooth borders and appeared to be dispersing.

Although pepsin activity is low in the stomach (29), proteolytic trypsin and chymotrypsin are present in the duodenal lumen and may disrupt the chains of casein and the individual micelles. Proteolytic activity (nitrocasein substrate) is present in the jeunums of suckling rats but has been shown to be about half of that in adult rats. Proteolytic activity in the distal portion of the small intestine in suckling rats is similar throughout suckling to adulthood (30).

**Milk Fat Globules in Duodenal Contents**

Milk fat globules in the central portion of the duodenal lumen were uniformly opaque and were smooth in outline. SEM showed their surfaces to be covered by folds or "ruffles" (Figure 12). TEM showed that there were few membranes surrounding these globules (3). The membrane fragments were in the lumen nearby (Figure 13). This suggests that the membrane fragments present on milk fat globules in the gastric lumen become detached from the droplets when the latter are in the duodenal lumen. Sodium deoxycholate has been used experimentally to remove the membranes from bovine milk fat globules (17) and therefore biliary secretion may be acting similarly within the duodenal lumen.

Bergener (5) reported in 1962 that long clefts appeared in milk fat globules in the duodenal lumen. It is likely that these long clefts resulted from polymerization changes during the methacrylate embedment.

Milk fat globules between the villi were irregular in shape by SEM (Figure 14) and had irregular outlines with many lucent indentations by TEM (Figure 15). This has also been reported by Ferlatte and Zeman (11). Fat globules deeper between the villi were more irregular than those near the villus tips (Figures 16 and 17) as was also noted by Bergener (5) in hamster intestine. This appearance is similar to that of chylomicrons (serum lipid droplets undergoing lipolysis) in vitro (6,7,8). Similar indentations in chylomicrons have been considered to be the sites of accumulation of the amphiphilic molecules with water resulting from lipolysis of triacylglycerol.
In the rat, pancreatic lipase levels are only one-sixth of adult levels during the first 10 days of suckling and rise to adult levels by day 20 (25). During suckling, milk fat globules trapped between the villi probably have more time to be hydrolyzed by the low lipase activity. The potent lingual lipase recently discovered by Hamosh et al. (15,16) may be significant particularly during the first 10 days of life in the suckling rat and in other mammals in which it may compensate for the low level of pancreatic lipase. Breakup of the fat globules is also aided by biliary emulsification (22). This emulsification and lipolysis produces macromolecular units which are then absorbed by the intestinal epithelial cells. The duodenal epithelial cells of suckling rats re-esterify the products of lipolysis to produce chylomicrons (2). Fat droplets associated with membrane fragments and cell organelles are also present in the duodenal lumen contents. These have originated from desquamated villus tip cells (Figure 18). It is probable that these will be reabsorbed.

It is concluded that ingested milk is converted to a curd within the stomachs of suckling rats. The linked casein micelles surround milk fat globules. There is no ultrastructural evidence of intragastric proteolysis. A granular form of casein is present as part of the intragastric curd of 24-hour-old rats. This form is not seen at 5 days or later ages. Its nature is not known. Within the duodenum casein particles separate and disperse. This probably results from the action of chymotrypsin. Milk fat globules within the stomach are surrounded by membrane fragments. Milk fat globules lose these membranes in the central duodenal lumen. Between the villi the milk fat globules show peripheral lipolysis. The absorbed triacylglycerol reappears within the duodenal epithelial cells.

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References


Discussion with Reviewers

M. Kalab: Is there a protease similar to calf rennet also present in the stomachs of newborn monogastric animals?
Author: No such enzyme has been identified. Parietal cells produce gastric acidity, but pepsin does not appear until weaning. Thus, in the rat, curd formation is probably a function solely of pH. The low level of gastric proteolysis conserves the lingual lipase present in gastric contents, but may be sufficient to produce liquefication of the curd at the pyloric antrum.

J. Glaser: What do you feel is the reason for some of the casein particles exhibiting electron dense areas as irregularly interconnected polygonal rings (Figure 6) while other opaque areas appear as parallel lines (Figure 7)?
Author: The interconnected polygonal rings seen in some casein particles probably represent those in which the particles have been sectioned through the centers, and therefore represent the internal structure of the particles. Casein particles with opaque parallel lines probably represent particles in which superficial sections have been cut. The opaque parallel lines are probably surface features.

J. Glaser: What do you feel is the reason for the change in electron opacity between fused casein micelles in the gastric curd and those unfused micelles in the duodenal lumen?
Author: The patterns in casein micelles in the gastric curd are probably lost by those in the duodenal lumen following exposure to the proteolytic action of pancreatic chymotrypsin.