Digestibility and Absorption of Protein in Infants

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Proteins in breast milk and infant formulas are often assumed to be well utilized by infants and to cover their amino acid requirements. While this is likely to be largely correct and while insufficient amino acid availability is unlikely, some aspects require further scrutiny. Several proteins in human milk are not well digested and can escape digestion, making a discussion of protein requirements of breast-fed infants necessary. In addition, processing and protein composition of formulas will affect protein digestibility, necessitating a reevaluation of the optimum protein intake of formula-fed infants. The increased use of metabolic indices in infants to evaluate the adequacy of different diets will also demand a better understanding of the physiological events in the gut that precede the amino acid levels in plasma.

DIGESTIBILITY OF HUMAN MILK PROTEINS

The ability of human milk proteins to serve as a source of amino acids ("nutritional protein") to the breast-fed infant has been an area of controversy. While it was earlier believed that human milk protein was very well digested and utilized, it has recently been argued that some of the proteins in human milk that resist digestion should not be included when calculating the amounts of protein and amino acids provided to the breast-fed infant (1). The truth is somewhere in between—not all proteins in human milk are well digested; on the other hand, even if a proportion of these proteins escape digestion and are found in the stool, this fraction is quantitatively minor (2).

Secretory IgA (SIgA) was detected in the feces of breast-fed infants over 20 years ago (3). This particular form of IgA, which is present in high concentrations in breast milk, particularly in colostrum, consists of two molecules of IgA linked together with two other molecules, the secretory component and the J-chain (3). This molecular arrangement renders SIgA uniquely stable against degradation by proteolytic enzymes (4). However, it should be emphasized that there is not absolute protection; with time, SIgA will ultimately be degraded by digestive enzymes. The extent of this degradation (i.e., gastric acid secretion and pH, pepsin and pancreatic enzyme output, and transit time) will be dependent on the maturity of the infant.
A study of the persistence of human milk proteins in exclusively breast-fed infants showed that three proteins could be detected in the stool in significant quantities: secretory IgA, lactoferrin, and α1-antitrypsin (5,6). The proportion of intact milk proteins found in the feces varied with the age of the infant; about 10% of the total protein intake of the breast-fed infant was found during the early neonatal period (0–1 month of age), while only 3% was found at 4 months of age (Fig. 1). That the excreted proteins originate from human milk and are not secretory products from bile, pancreatic fluid, or intestinal cells is evident from the fact that only negligible quantities of these proteins are found in the feces collected from infants fed formulas based on cow's milk (7). Some early studies also describe lysozyme in the stool of infants and children (8); however, the origin of this lysozyme was not known.

The presence of intact lactoferrin in the stool of infants has been used to suggest a physiological role for lactoferrin in the gastrointestinal tract of breast-fed infants (9). It has been proposed that lactoferrin facilitates iron absorption (10), inhibits the growth of intestinal pathogens (11), and stimulates the growth of the intestinal mucosa (12), all of which require the presence of intact lactoferrin within the gut lumen. In vitro experiments have shown that lactoferrin has an unusual stability against proteolytic enzymes; however, at low pH, lactoferrin will eventually be degraded (13).

The survival of some breast milk proteins may not only be explained by the stability of these proteins against proteolytic attack, but also by the presence of protease...
inhibitors in human milk that may inhibit some of the proteolytic capacity of the infant (6,14). In particular, the finding of considerable quantities of α₁-antitrypsin in breast milk and the stool of breast-fed infants (6) suggests that this protein may inhibit part of the trypsin activity in the gut. As much as 0.3-0.6 mg of α₁-antitrypsin per milliliter was found in early human milk, and even if concentrations were lower in mature milk, the protein was still detectable after 3–4 months of lactation. The proportion of α₁-antitrypsin in the stool decreased during lactation: during the first weeks 50–60% was found intact, while 20–30% was found at 3–4 months of age. In vitro digestion experiments showed that α₁-antitrypsin on its own was digested, while α₁-antitrypsin together with trypsin resisted digestion by pancreatic enzymes. Other protease inhibitors have also been detected in breast milk, such as α₁-antichymotrypsin and antielastase (14); however, the concentrations of these inhibitors are considerably lower than that of α₁-antitrypsin and their quantitative role may be less significant.

The digestibility of human milk proteins has also been studied in preterm infants (15,16). It is obvious that the premature infant's capacity to digest milk proteins is more limited than that of the term infant: not only does a larger percentage of proteins resist proteolysis, but other milk proteins, such as lysozyme and serum albumin, are also found in their stools (Table 1). When the infants were fed a combination of preterm milk and formula, a higher proportion of lactoferrin (13% vs 5%) and lysozyme (18% vs 3%) was found in the feces (16). Thus the presence of other proteins in the gut of the preterm infant may limit proteolysis of breast milk proteins. It should be emphasized that the immunological techniques used to detect and quantitate human milk proteins also recognize larger fragments (epitopes) of these molecules. Chromatographic separation of soluble proteins from the feces showed that part of the proteins is present in intact form, but that partial proteolysis creates some larger peptides (Fig. 2). It is possible, however, that some of these larger fragments still exert some biological activity; for example, the intestinal lactoferrin receptor will bind larger fragments of human lactoferrin (17).

<table>
<thead>
<tr>
<th>TABLE 1. Intake and fecal excretion of human whey proteins*</th>
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<tr>
<td>PTM group</td>
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<tr>
<td>Intake</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
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<tr>
<td>Serum albumin</td>
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<tr>
<td>Lactoferrin</td>
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<tr>
<td>Lysozyme</td>
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<td>Secretory IgA</td>
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* Values represent mean ± SD mg/kg/d.
* Calculated as a percentage of intake.
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DIGESTIBILITY OF FORMULA PROTEINS

Infant formulas normally contain higher levels of protein than human milk (18). The rationale for these higher levels of protein has been that they will assure the infant of an adequate supply of amino acids from a protein source with assumed lower digestibility. However, the digestibility of heat-treated milk proteins should be relatively high and it has been suggested that the commonly used protein level in formula of 15–20 g/liter may be unnecessarily high (19,20). This notion was supported by observations that metabolic indices, such as plasma amino acids and blood urea nitrogen, are quite different in formula-fed infants than in breast-fed infants.

If the protein level of infant formula is lowered, the protein and amino acid intake of the formula-fed infant will by definition become closer to the requirement of the infant, at the same time narrowing the "safety" margin. In this situation, the protein quality of formula becomes more critical. All formulas are exposed to heat treatments which vary with regard to both temperature and duration. There has been very limited consideration of the significance of using different heat treatments for production of liquid formulas ("in-can" sterilization, >110°C, extended time periods) and powdered formulas (spray-drying, 60°C, brief exposure). More recently, ultrahigh temperature treatment (UHT, >130°C, 3–5 s) has become more common for production of
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TABLE 2. Gastric acid and pepsin output at different ages

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<th>Acid (mEq/h)</th>
<th>Pepsin (mg/h)</th>
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<tr>
<td>Infant (1 day)</td>
<td>13.2</td>
<td>0.18</td>
</tr>
<tr>
<td>Infant (3–8 days)</td>
<td>0.06</td>
<td>0.21</td>
</tr>
<tr>
<td>Infant (10–11 days)</td>
<td>0.12</td>
<td>0.46</td>
</tr>
<tr>
<td>Infant (14–17 days)</td>
<td>0.19</td>
<td>0.88</td>
</tr>
<tr>
<td>Infant (67–110 days)</td>
<td>0.47</td>
<td>1.34</td>
</tr>
<tr>
<td>Children (4–8 yr)</td>
<td>4.9</td>
<td>18.5</td>
</tr>
<tr>
<td>Adults</td>
<td>13.1</td>
<td>41.9</td>
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ready-to-feed formula. Besides the heat treatment, the protein composition (casein-predominant vs whey-predominant) of the formula may be expected to affect protein digestibility (21).

The digestibility of milk proteins can be assessed by in vitro methods or in vivo, using experimental animals or human infants. The in vitro methods usually employ a combination of proteolytic enzymes such as pepsin, trypsin, or chymotrypsin, or, as in some studies, gastric and/or duodenal aspirates from infants in order to mimic more closely the conditions of the infant’s gastrointestinal tract. An important consideration in all these methods is the time factor; with time, most proteins will be digested; however, passage through the gastrointestinal tract in infants is rapid and the time of exposure to enzymatic digestion is short. In addition, gastric acid secretion is relatively low (Table 2), making the stomach pH comparatively high and limiting pepsin activity (22). Development of the exocrine pancreas is not complete at birth (23), making the activities of several pancreatic enzymes lower than normal (24,25).

We have assessed the digestibility of milk proteins in infant formulas that had been exposed to various types of heat treatment (26,27). For the identical product, UHT-treated formula showed a significantly higher protein digestibility than formulas that were powdered (spray-dried) or sterilized (84% vs 72%). When comparing products that are present on the market in both powdered and sterilized form, the powdered products consistently showed higher protein digestibility, although the difference was less pronounced than that found for the UHT products (Table 3). Thus the more intense the heat treatment, the lower the protein digestibility. As an index of the heat treatment that the formulas had been exposed to, we analyzed available lysine in the product. Powdered products were consistently found to have significantly higher levels of available lysine, although the magnitude of the difference was not large. To explore the cause of the different digestibilities found, we subjected the formulas to various pH exposures, centrifugation, gel filtration, and gel electrophoresis. By using these techniques, we were able to study protein solubility and the distribution of protein between the fat, the soluble fraction, and the insoluble fraction. We found protein–protein and protein–lipid interactions in all formulas tested and these were more pronounced in the sterilized products than in powdered and UHT
products. These results support the hypothesis by Rowley & Richardson (28) that the composition of the lipid–fluid phase interface in milk is determined by the temperature and the duration of heat exposure used during processing. In addition, they stated that if the pH reaches the isoelectric point of the protein (e.g., pH 4.6 for bovine caseins), proteins are less charged and more hydrophobic and can therefore interact with uncharged lipids. We found that enhanced protein–lipid interactions occurred in the pH range 4.0–5.0, which is similar to the pH in the stomach of young infants (29,30). In particular, casein and β-lactoglobulin in sterilized products was found associated with lipids at this pH range. This was further documented by gel filtration and gel electrophoresis, showing that few proteins were soluble at this pH and also that casein and β-lactoglobulin were associated with the lipid fraction. Since heat treatment opens up disulfide bridges within proteins such as β-lactoglobulin and κ-casein, reactive sulfhydryl groups may now form covalent bonds with other components. It has been suggested that the formation of such bonds is responsible for the lower protein digestibility. Thus the new disulfide bonds formed and the modifications of some amino acid residues (e.g., lysine) by Maillard reactions may limit the accessibility of proteolytic enzymes and consequently, protein digestibility.

Individual milk proteins have different capacities to resist proteolysis. Jakobsson et al. assessed the digestibility of some human milk and bovine milk proteins in vitro by using either duodenal juice from infants (31) or trypsin and elastase (32). They found that human lactoferrin was very slowly digested, while bovine casein was degraded rapidly. Interestingly, human α-lactalbumin was found to be more slowly digested than bovine α-lactalbumin, even if their structure and composition are very similar.

The suckling rat pup model has also been used to assess protein digestibility in vivo (33; Lönnerdal et al., unpublished). At this developmental stage, the rat pup's capacity to digest proteins has not yet been fully developed and transit through the
gastrointestinal tract is rapid, limiting the time for digestion to occur. We therefore believe that this is a good model for the human infant and, at the least, it allows us to compare the digestibility of proteins in formulas that have been processed in various ways. It should be noted, however, that the older rat (weanling or adult) has a very efficient digestive "machinery"—differences among various formulas are rarely observed and digestibility is always very high (34). In our model (LönnérDAL et al., unpublished) diets are intubated into the stomach of fasted pups and the animals are killed at various time points (1-4 h) after the dose has been given. The stomach and the small intestine are perfused separately and the perfusates are then separated into soluble and insoluble proteins by centrifugation. Nitrogen and nonprotein nitrogen are analyzed in all fractions. By this method we found that proteins in UHT-treated and powdered products were most rapidly digested, while sterilized formulas were more slowly digested (Fig. 3). Protein digestibility of soy formulas was also found to be slow. Although most formula proteins were digested with time, our results suggest that when transit time is short, as in newborn infants, slow digestibility may result in lower amino acid availability to the infant.

**IMPLICATIONS OF DIFFERENCES IN PROTEIN DIGESTIBILITY IN INFANT NUTRITION**

It is obvious that a lower digestibility of proteins in infant formula will affect the amount of amino acids and smaller peptides available for absorption by the infant.
Whether the differences in digestibility found for formulas that have been exposed to different heat treatments are large enough to have a significant quantitative effect in infants remains to be studied. However, it appears prudent to attempt to optimize protein utilization from infant formula, as undigested fragments may affect utilization of other nutrients (see below). In addition, knowledge about protein digestibility is important when deciding on the appropriate level of protein to use in formula. For example, fasting levels of plasma amino acids are often used to evaluate protein and amino acid adequacy from different formulas in clinical studies in infants. In such studies, the type of heat treatment is rarely presented, only the brand name. Thus data may be derived from sterilized or powdered products and the results may be as dependent on the degree of heat treatment as on the protein level or composition being studied. An important illustration of this is given by the recent study of Raihā et al. (personal communication), who gave premature infants either formulas exposed to different heat treatments or human milk. Plasma amino acid profiles were taken at hourly intervals and protein intake was normalized between the diets. In infants fed human milk, plasma amino acids rose quickly after a meal and then decreased rapidly and reached the baseline after about 2 h. The plasma amino pattern of infants fed UHT-treated formula was similar to that of breast-fed infants, while the peak and decline in amino acids were somewhat slower in infants fed powdered formula. More dramatically, however, infants fed sterilized formula had a much slower increase in plasma amino acids, and the levels had not returned to baseline even 4 hours after feeding. This observation has two important implications: (a) protein digestibility from formula in infants is affected by the degree of heat treatment; and (b) the slower protein digestibility of sterilized formula makes the fasting plasma amino acid pattern an unreliable diagnostic tool for assessing protein quality.

Lower protein digestibility causes a higher percentage of larger peptides and proteins to persist longer in the small intestine. Since some of these proteins/peptides can bind minerals and trace elements, they may limit the uptake of such essential nutrients, either at the site of their maximum absorption (often the duodenum), or within the entire intestine if they ultimately leave for the colon. We have shown both in human studies (35) and in experimental animals (36) that casein-predominant formulas result in lower zinc bioavailability than whey-predominant formulas, presumably because of less complete digestion of casein. However, it should also be recognized that some peptides formed during digestion of casein, the so-called casein phosphopeptides, may have a positive effect on calcium absorption (37,38). Furthermore, we have shown in our suckling rat pup model that trace element bioavailability was highest for UHT-treated and powdered products, but lower from sterilized products (Lönnerdal et al., unpublished). Thus protein composition and protein digestibility can affect mineral and trace element bioavailability from formulas.

REFERENCES

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DISCUSSION FOLLOWING THE PRESENTATION OF DR. LÖNNERDAL

Dr. Räihä: I think this is the beginning of a very important new area concerning the manufacture of infant formulas. What Dr. Lönnerdal has shown *in vitro* and partly in his rat studies, and which we have been able to confirm in the premature infant *in vivo*, is that human milk has a very different pattern from formula when you look at the speed of absorption of amino acids and also at the height of the postprandial plasma amino acid concentration curve. We don’t really know what this means. We can only say that some formulas are more like human milk and others are less like it when you look at these variables. However, I think these findings have clinical and technical implications. For example, the speed at which the amino acids increase in the plasma and then return to baseline may have an effect on release of gastric hormones, insulin, and so on, and this of course can affect the baby. Also, you have to be very precise about the time of sampling. We have compared studies in premature infants given either human milk or formula in the same quantities. If you take preprandial samples, usually about 2½–3 hours after the last feed, it is always clear that the infants fed human milk have lower plasma amino acid levels than those of infants fed formula.

I think the explanation for this may be that in the formula-fed infants the amino acids have not yet come down to baseline. We must remember this when we refeed the baby, because we may be pushing up the levels higher and higher without allowing them time to return to baseline. So I think there are many clinical implications related to these findings.

Dr. Guesry: The smoother postprandial amino acid curve with casein-predominant than with adapted whey formulas is in keeping with work by Jacques Senterre showing that gastric emptying was much slower with casein formulas than with adapted whey formulas. My second comment is for our colleagues from developing countries who may follow a routine of pasteurizing their powdered formulas in the autoclave, perhaps for as long as 1 hour at high temperature. This is likely to harm the protein, which was originally of good quality and well absorbed.

I have a question relating to the quantitative assessment of protein. It used to be assumed when calculating the protein content of human milk that the functional proteins, such as
secretory IgA, lactoferrin, and lysozyme, were not digested, and only the true nutritional protein was taken into account. However, you have now shown us that 85-90% of these functional proteins are indeed digested and could be used for nutrition. I think it is time that we reassessed the quantity of protein in human milk so that we can judge the right quantity for infant formula.

Dr. Raihā: No one has ever claimed that the protective proteins are not absorbed at all. But even if we were to assume that only between 3% and 5% of the total proteins in human milk are not absorbed, we would still find that the nutritional protein concentration in human milk is very low compared to all the formulas on the market today. Even if you take into account the amount of nonabsorbed whey protein, many formulas today still have almost twice as much nutritionally available protein as human milk.

Dr. Rassin: I wonder if another way to interpret the curves of the total amino acids is that the amino acid concentrations are regulating the feeding patterns of the infant, because you get much more frequent feeding in breast-fed infants than in formula-fed infants. Perhaps what really happens is that you see different responses to those amino acids.

Dr. Raihā: The study that we showed was based on one bolus feed.

Dr. Rassin: I can understand that, but in a normal situation your breast-fed baby is going to feed maybe every 2 or 2½ hours, while frequently in the nursery we have no problem getting formula-fed babies to go for several hours between feeds. What I am saying is that I wonder whether these amino acids are playing a role in the regulation of these feeding patterns through an influence on hormone release and brain response.

Dr. Axelsson: Dr. Lonnerdal, what was the composition of the cereal-based formula you used that was poorly digested? Was it based only on cereal proteins?

Dr. Lonnerdal: It was based on a mixture of rice and barley. We have also looked at mixtures of cereals and milk which are quite commonly used, and their digestibility is not much better. Digestibility is slower for such formulas and this reemphasizes that we should not start to feed cereals to infants at too young an age.

Dr. Heine: Is anything known about the enzymes that split off the carbohydrate side chain of glycoproteins and food proteins such as casein? What enzyme is it?

Dr. Lonnerdal: Some of the brush border enzymes can have this role. The digestibility of the protein is important because the activity of some of the carbohydrate metabolizing enzymes may be dependent on how much of the polypeptide has been split to allow access to the branch structures.

Dr. Heine: You mentioned the loss of bioavailability of lysine during heat treatment. Are there any other amino acids that lose bioavailability under these conditions? What about tryptophan, for instance?

Dr. Lonnerdal: This has been reported in the literature; serine and threonine can also be affected but I am not aware of any very good methods for easily detecting these effects.

Dr. Raihā: Hydrolyzed and partially hydrolyzed formulas are becoming more and more popular, and are sometimes used in premature infants. According to your data, these should be absorbed very differently and probably much faster, so there may be marked physiological effects.

Dr. Lonnerdal: It is quite likely that these formulas may have marked effects on hormonal responses following rapid surges in substrate levels in the blood, but I have not seen much work on this.

Dr. Marini: I would like to comment on this. We have studied gastrointestinal hormones in full-term babies fed with hydrolyzed formulas. In general, the responses were much the
same as with human milk, but we found differences in enteroglucagon and pancreatic polypeptide inhibitors. It is easy to explain this because they don't need pancreatic hydrolysis. In another study we looked at gastric function and found that basal and maximum acid output with hydrolyzed formula was more similar to that found with human milk than with artificial formulas.

**Dr. Guesry:** If we consider that gastric emptying is an index of digestibility, then in the curve which starts with low digestibility for raw cow's milk and proceeds to the best digestibility or best gastric emptying for human milk, hydrolyzed formula is closest to human milk, with a gastric emptying time of less than 2 h.

**Dr. Räihä:** You showed the difference between human milk and human milk to which formula was added. We often supplement human milk with bovine proteins when feeding premature infants. Do you know of any studies looking at protein digestibility in such cases?

**Dr. Lönnherdal:** We have looked at some of these human milk fortifiers and they are remarkably insoluble. They are much more insoluble than milk formulas and I have the feeling that the processing of some of these cow's milk proteins is quite different. When you prepare a whey protein milk powder there are many other substances present which help to stabilize the mixture during spray drying. In the case of milk fortifiers, consisting only of proteins, the proteins become very hydrophobic. If you centrifuge fortified human milk you find a pellet at the bottom consisting of the fortifier proteins. The various commercial brands that we have investigated have all been more or less totally insoluble.

**Dr. Pettifor:** We are becoming increasingly aware of the role of the large bowel in carbohydrate absorption. Is there any evidence that bacterial flora play a role in protein digestibility?

**Dr. Uauy:** This is an interesting question and has particular relevance to urea. Urea is secreted into the upper gut, and flora definitely play a role in urea recycling; there is also a possibility that non-absorbed proteins are modified by the intestinal flora. I know that Alan Jackson is currently looking at the effect of the gut flora in producing some of the essential amino acids and we shall probably have interesting surprises relating to the role of the flora and the colon modifying the amino acid supply. In the ruminants the role of the flora in determining the amino acid supply is well established.

**Dr. Heine:** We have given 15N-labeled protein into the colon of babies with colostomies and we observed 80% nitrogen absorption. Since the colon has no proteolytic enzymes, we must assume that the bacterial flora are splitting this protein down to the amino acid label to allow its absorption. We have run the same experiments with bacterial nitrogen and found the same absorption rate.

**Dr. Lönnerdal:** Were those studies done in preterm infants?

**Dr. Heine:** There were preterm infants among the subjects.

**Dr. Räihä:** How much protein are we talking about? Is it a substantial amount or of only minor importance?

**Dr. Heine:** The amounts absorbed in our studies were of the order of grams rather than milligrams. This might be important in the malnourished infant when the protein supply is low. The mode of absorption from the colon is not quite clear. It might be that some protein is digested within macrophages and other cells, but we can't explain the high absorption rate in any other way than that there is digestion by the intestinal flora.

**Dr. Marini:** Dr. Lönnerdal, you used 10 to 14-day-old rats for your study. Wouldn't it have been better to have used neonatal guinea pigs? It is said that neonatal guinea pigs are more similar to human beings.
Dr. Lönnerdal: It is quite possible that it would have been better. However, the rat produces very large litters and is a good laboratory animal for screening tests even if it may not be the ideal model. When we see relevant differences we can move on to studies in primates or in premature infants.

Dr. Ali Dhansay: I would like to ask a question related to lipid protein interactions. There are situations when one uses medium-chain triglycerides as an additive to formula feeding. Have you any suggestion as to when and how much one should add with respect to digestibility of proteins in the formula itself?

Dr. Lönnerdal: My feeling is that it would not matter much for this phenomenon whether there are medium-chain triglycerides or long-chain fatty acids present; you would most likely see the same type of hydrophobic interaction regardless of chain length.