# The amino acid composition of human milk - towards determining the amino acid requirements of the human infant

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This thesis is dedicated to God,
for without him it would not exist,
both in concept and in completion.

# ABSTRACT

The overall aim was to determine the digestible amino acid composition of human milk. The gross amino acid composition of human milk was corrected for digestibility using true ileal amino acid digestibility coefficients for human milk determined in the piglet. The digestible amino acid composition of human milk was used to evaluate protein quality in a commercial infant formula.

In the first part of the study the piglet was evaluated as a model animal for studying aspects of protein digestion in human infants. Three-week-old male piglets and three-month-old male human infants were bottle-fed an infant formula over a 17 day balance study which included a 10 day total faecal collection period. Small but statistically significant differences between the piglets and infants were found for the apparent faecal digestibility of dietary dry matter, organic matter and total nitrogen. The faecal digestibilities for most of the amino acids, however, were not significantly different between the species. It was concluded that the digestion of protein, to the end of the gastrointestinal tract, appeared to be similar in the two species.

For application of an ileal digestibility assay, it is necessary to assume that amino acids are not absorbed in significant amounts posterior to the ileo-caecal junction. An experiment was conducted, therefore, to determine whether lysine and/or methionine, two dietary essential amino acids, were absorbed in nutritionally significant amounts from the large intestine of the three-week-old piglet. Piglets, surgically prepared with simple catheters which allowed infusion into the proximal colon, were given one of two milk-formula diets which were deficient in either lysine or in the sulphur amino acids, yet were balanced for all other amino acids. An isotonic solution containing the respective deficient amino acid or physiological saline was infused via the catheter at each feeding. Total daily excretions of urinary urea and total nitrogen were determined. There were no significant differences in urinary nitrogen metabolite excretion for piglets infused with amino acids compared with those infused with saline. Lysine and methionine did not appear to be absorbed in nutritionally significant amounts from the proximal colon of the milk-fed piglet.

Two experiments were conducted to develop a method for accurately determining the amino acid composition of human milk. In the first, a non-linear model, that describes the simultaneous processes of amino acid yield and decay that occur during acid hydrolysis of a protein prior to amino acid detection, was used to regress data derived from multiple hydrolysis intervals. Most of the amino acids

underwent some degree of loss during hydrolysis. Of particular note was the loss rate for cysteic acid, which was greater than that found for serine. Using the routine duplicate sampling system, a non-linear regression including 10 hydrolysis intervals resulted in a mean amino acid recovery of 100% and provided an acceptable compromise between accuracy and the cost of analysis. In the second experiment, the non-linear model was modified to account for samples, such as human milk, having amino acids in free form prior to hydrolysis. The original and new models were compared. A biological sample (human milk) was hydrolysed in acid for multiple hydrolysis intervals. As in the previous experiment, most of the amino acids (and in particular, cysteic acid) underwent some degree of loss during hydrolysis. It was concluded that using the original model to analyse data obtained from hydrolysis of a sample containing protein and free amino acids will not lead to the introduction of any large bias in the determination of amino acid composition. The modified model, however, is more accurate for application where a sample contains both protein-bound and free amino acids.

In the penultimate experiment of the study, human milk was collected from women in their 10th-14th weeks of lactation, and was analysed for amino acids with correction for losses of amino acids during acid hydrolysis, using the model parameters determined in the earlier experiment. The mean amino acid composition of the human milk was similar to previously reported estimates, though the cysteine content of the human milk was 20% higher than the mean literature estimate. True (corrected for endogenous amino acid excretion) ileal amino acid digestibility coefficients for human milk, determined in three-week-old piglets fed human milk, ranged from 81-101% with threonine (86%) being the least digestible essential amino acid. The overall digestibility of amino acid nitrogen was 95%. When the true ileal digestibility values were used to correct the amino acid composition of human milk, the pattern of amino acids absorbed from human milk was different compared to the currently recommended dietary pattern of amino acids for the infant.

In the final study, true ileal amino acid digestibility coefficients for a commercial infant formula were determined using the three-week-old piglet. Coefficients ranged from 95% for lysine to 103% for arginine, indicating near-complete digestion of the protein in the infant formula. The profile of absorbed amino acids for the infant formula was compared with that for human milk to evaluate protein quality in the infant formula. It was concluded that the protein in the formula was of high quality.

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# GENERAL INTRODUCTION

From the very earliest days of man's existence, human infants would have been reared almost exclusively on human milk in the first few months of life. It seems reasonable to assume that during this time, human milk evolved to match the nutritional requirements of the human infant and to promote survival of the species. Certainly, human milk appears to be uniquely adapted to the infant's physiological needs, affording the infant both nourishment and immunological protection.

The nutrient composition of human milk is thought to reflect the infant's nutrient requirements, and as such, the amino acid composition of human milk is used to determine amino acid requirements for infants. Using the gross amino acid composition of human milk for this purpose may not be appropriate, however, as several of the proteins in human milk have an immunological function and these proteins are thought to resist, at least partially, digestion in the infant's gastrointestinal tract. The digestible rather than gross amino acid composition of human milk, therefore, may be a better reflection of the infant's amino acid requirement.

The main objective of this study was to determine the digestible amino acid composition of human milk, as a contribution towards determining the amino acid requirements for human infants. Central to this objective were two factors: (1) accurate determination of the gross amino acid composition of human milk, and (2) determination of ileal amino acid digestibility coefficients (corrected for endogenous amino acid excretions) which would be used to correct the gross amino acid composition of human milk for digestibility.

The first aim of the study was to evaluate the piglet as a model animal for studying aspects of protein digestion in the human infant. As the ileal assay relies on the assumption that amino acids are not absorbed from the large intestine, the second aim of the study was to investigate whether amino acids are absorbed in nutritionally significant amounts from the large intestine of the milk-fed piglet. Thirdly, a methodology for accurately determining the amino acid composition of human milk was proposed and tested. The fourth aim was to determine the digestible amino acid composition of human milk and finally to apply the determined digestible amino acid composition of human milk in evaluation of the protein quality of a commercial infant formula.

# CHAPTER 1

# REVIEW OF LITERATURE

## 1.1 INTRODUCTION

At birth the neonate experiences a sudden change from parenteral (placental) to enteral nutrition. Mammalian species have evolved to accommodate this change by the female of the species nourishing her offspring with milk, the secretion of the mammary gland. The most primitive mammary glands appear in the egg-laying mammals, where the glands consist of two abdominal lobes without true teats (Raynaud, 1961). Marsupials, often regarded as being more primitive than eutherian mammals such as man, have attained the most sophisticated mammary development of all mammals (Renfree, 1981). The neonate is born at an early foetal stage, travels unaided to the pouch where it attaches to a teat and begins to suckle continuously. It remains in the pouch through a period which corresponds approximately to the latter portion of intrauterine gestation in eutherian mammals. The nipple and gland grow, along with the suckling young, and the composition of the mammary secretion changes markedly with growth. It is fascinating to note, that the female marsupial can suckle both a newly born neonate and a well-developed offspring. The two young, at very different developmental stages, continue to suckle on glands which are also at very different developmental stages, receiving milks of quite different composition (Neville and Neifert, 1983).

Milk can be described as a complex secretory fluid containing lipids in emulsion, proteins in colloidal dispersion and various organic and inorganic constituents in aqueous solution (Oftedal, 1984). In addition to its obvious role in nutrition of the neonate, milk is involved in the development of the neonate's gastrointestinal tract (Widdowson, 1985), and in the establishment of beneficial gut bacteria (Roberts *et al.*, 1985). Another well documented role of milk is the immunological protection it bestows upon the neonate through the action of several proteins among other components (McClelland *et al.*, 1978).

The composition of milk varies considerably between different mammalian species and can be related to the suckling frequency, the external environment, and the growth rate of the neonate. High-solute milks are found in mammals that

provide large infrequent feeds to their young, such as the rabbit that only suckles once every 24 hours (Oftedal, 1984). High calorific density, principally due to the fat concentration of the milk (blue whale milk contains 50% fat), is usually related to low environmental temperatures, when the young have to develop and maintain a thick layer of insulating subcutaneous fat.

In general, the protein content of milk varies with the rate of growth of the offspring across species. This approximate relationship was originally suggested by Bunge in 1874 (cited by Blaxter, 1961), who correlated the protein content of different mammalian milks with growth rate, as indicated by the time taken by the neonate to double its birthweight. For example, the rabbit pup doubles its birthweight in less than 6 days, and rabbit's milk contains more than 14% protein to accommodate this rapid accretion of body protein. In contrast, the human infant may take in excess of 180 days to double its birthweight while consuming human milk with a protein content of less than 1%. The variation in protein content does not correlate well with relative growth rate (Oftedal, 1986), however, with the exception of the primates who have both low protein levels in their milk and a low relative growth rate.

Compared to the milk from other mammalian species, human milk has the lowest concentration of protein, calcium, and phosphate and the highest concentration of lactose (Jenness, 1979) and, thus is appropriate for a slow growing, continuous-contact species like man. In biological terms, man's chief characteristic is his large, complex brain, and human milk is rich in those nutrients (e.g. lactose, cysteine, cholesterol, thromboplastin) known to be important for brain growth and maturation (Casey and Hambidge, 1983).

In 1776, Professor Nils Rosen Von Rosenstein wrote: "*A child ought to get a sufficient quantity of good nourishment if it is to thrive well. The best food for it is, no doubt, the mother's milk*" (cited by George and DeFrancesca, 1989). This view that human milk is uniquely adapted to the nutritional requirements of the infant is now widely accepted, and the composition of human milk is used as the standard for infant nutrition during the first few months of life.

There would appear no need, therefore, to determine nutrient requirements for infants, given that breast-feeding of the infant is recommended. When a mother cannot or chooses not to breast-feed, however, a suitable human milk substitute is necessary. In addition, very small premature infants, critically ill infants, and infants with metabolic diseases often need to be taken off breast milk and fed specialised

formulas. A better understanding of the nutrient requirements of infants aids in the development of formulas best suited to the situations described above.

Although the nutrient composition of human milk is thought to reflect the infant's requirements using the gross protein composition of human milk may not be appropriate. Milk has many functions other than the provision of nutrients, including immunological protection of the infant. The majority of the immune factors in milk are proteins and for these proteins to function it is anticipated that they would remain intact within the infant's gastrointestinal tract, and as such, would not contribute amino acids for nutrition. Therefore, the absorbed amino acid composition rather than the gross amino acid composition of human milk may better reflect the infant's requirement for amino acids.

The following discussion examines the different methods available for determining the amino acid requirements of healthy, full-term infants. Given that the majority of healthy infants will be exclusively breast-fed for up to 6 months of age only, the following discussion pertains to this age group. The approach of using the gross amino acid composition of human milk as a standard upon which to base estimates of the infant's requirements is discussed in detail, particularly with regard to the presence of biologically active proteins in human milk that may not be of any nutritional benefit to the infant. The possibility of correcting the amino acid composition of human milk for digestibility is examined, as is the use of the piglet as a model animal for studying aspects of protein digestion in human infants.

## 1.2 DEFINING THE PROTEIN REQUIREMENT OF INFANTS

An infant's requirement for protein is best described as a requirement for the individual amino acids that make up the protein.

In a series of classical nitrogen (N) balance studies by Rose and colleagues, summarised by Rose (1957), eight amino acids were identified as being dietary essential in man (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) with the remaining amino acids classified as non-essential. In a similar series of studies conducted with infants (Snyderman *et al.*, 1955; Pratt *et al.*, 1955; Snyderman *et al.*, 1959a; 1959b), the same general conclusions were reached as to which amino acids were essential for the infant. More recent studies suggest, however, that the classification introduced by Rose lacks flexibility and alternative systems of classification have been proposed (Irwin and Hegsted, 1971; Jackson, 1983;

Laidlaw and Kopple, 1987). Jackson (1983) discussed the essentiality of amino acids based on the ability or inability of the body to synthesise all or part of an amino acid. As such, the amino acids can be classified into groups determined by whether or not the carbon skeleton or amino group is essential. Laidlaw and Kopple (1987) agreed with the biochemical and metabolic classifications suggested by Jackson (1983), but added that the amino acids should be grouped according to the ability of the organism to synthesise the individual amino acids in healthy and diseased states, as well as in different physiological circumstances. In summary, therefore, amino acids can be classified as either essential, conditionally essential or non-essential.

The list of conditionally essential amino acids required by infants has increased as data related to biochemical development in the neonate have been added to the original information from growth and N balance studies. Of particular interest in relation to the human infant are histidine, the amino acids involved with urea production, the aromatic amino acids and the sulphur-containing amino acids. Histidine is considered essential for normal growth in infants (Snyderman *et al.*, 1963), yet histidine essentiality in adults is still in question (Laidlaw and Kopple, 1987; National Research Council[1], 1989; Pellet, 1990). Arginine may also be conditionally essential in the infant (Rassin, 1989). This is based on the observations that although the enzymes required in the urea cycle are present in foetal liver tissue, when the activity of each enzyme is expressed as a proportion of adult hepatic activity, the enzymes involved in arginine synthesis have the lowest relative activity (Rassin, 1989). This may explain the increased need for arginine in infants on total parenteral nutrition (Heird, 1981), although Snyderman *et al.*, (1959c) found no evidence for an arginine requirement in infants based on the observation that an arginine-free diet caused no deterioration in health, weight gain or N balance in three newborn infants.

The infant's requirements for the aromatic amino acids, phenylalanine and tyrosine are more difficult to determine. While phenylalanine is accepted as being essential, tyrosine can be synthesised from phenylalanine. The inclusion of tyrosine in the diet, however, can exert a sparing effect on the dietary phenylalanine requirement. As a result, tyrosine has traditionally been considered as a conditionally essential amino acid. An inconsistency exists with regard to the infant's requirement for tyrosine. There is evidence to suggest that the infant may have a limited capacity to catabolise tyrosine rather than for synthesising tyrosine from phenylalanine. For

[1]*Hereafter NRC (1989)*

example, infants fed formulas which have excessive amounts of the aromatic acids, exhibit large increases in plasma tyrosine concentrations indicating a reduced ability to catabolise tyrosine. In contrast, preterm infants given a parenteral solution containing abundant amounts of phenylalanine and little or no tyrosine, showed decreased plasma tyrosine levels, impaired N retention and impaired weight gain (Snyderman 1971), suggesting a dietary requirement for tyrosine in the infant due to limited synthesis of tyrosine from phenylalanine. These observations imply that the balance between adequate and excessive amounts of the aromatic amino acids is delicate in the infant.

Taurine is one of the most abundant free amino acids in the human body, although it is not incorporated into protein (Jacobsen and Smith, 1968; Hayes and Sturman, 1981). Taurine has several biological functions, the best demonstrated of which is in the formation of bile acid conjugates. The initial concern as to whether taurine was essential arose because of the extremely low activity of the hepatic enzyme responsible for catalysing taurine synthesis in humans compared to that in rats (Gaull *et al.*, 1977). As taurine is not incorporated into protein, N balance studies may not demonstrate a response to varying levels of taurine in the diet (Laidlaw and Kopple, 1987), making it difficult to determine whether taurine is essential in the human infant. In addition, relatively large whole-body taurine pools may be difficult to deplete in short term studies, particularly since the turnover of taurine is slow (Laidlaw and Kopple, 1987). Human milk is rich in taurine compared to cow's milk (Blanc, 1981). Consequently, whether a taurine deficiency occurs in infants that are formula-fed has been extensively investigated (Räihä *et al.*, 1976; Gaull *et al.*, 1977; Rassin *et al.*, 1977; Rigo and Senterra, 1977; Järvenpää *et al.*, 1983a; Järvenpää, 1983b; Rassin *et al.*, 1983). Most studies found no effect of a taurine-free diet on the infant's clinical or nutritional status in comparison with infants fed either taurine-supplemented formulas or human milk. It would appear therefore, that when provided with a diet deficient in taurine, the infant's metabolism quickly adjusts bile salt conjugation from predominantly taurine conjugation to predominantly glycine conjugation (Encrantz and Sjovall, 1959; Poley *et al.*, 1964), thereby minimising body losses of taurine. This may in part explain the lack of a demonstrable effect of dietary taurine deficiency on growth and metabolic indices in the infant.

Although cysteine is not considered essential in the adult, the requirement being met by the metabolic conversion of methionine to cysteine, it has been

suggested that cysteine may be essential in infants. In the liver of foetuses (Sturman *et al.*, 1970; Gaull *et al.*, 1972), pre-term, and new-born term infants (Sturman *et al.*, 1970), a low or undetectable level of cystathionase (also known as cystathionine τ-lyase, and essential in the conversion of methionine to cysteine) activity has been observed. Snyderman (1971) and Pohlandt (1974) both reported that in pre-term and term infants receiving a parenteral solution with a reduced level or no cysteine, plasma cysteine levels decreased rapidly or remained very low. When the reduced plasma cysteine levels were not improved by the addition of methionine to the parenteral solution it was concluded that the enzymatic conversion pathway was limiting, making cysteine conditionally essential. Zlotkin and Anderson (1982), questioned the essentiality of cysteine, however, when they failed to detect improved N retention in full-term infants when cysteine was added to a total parenteral nutrition regimen previously devoid of cysteine.

Glycine has also been suggested as a conditionally essential amino acid in the infant (Jackson, 1981; Jackson, 1989), although some of the results from these studies may reflect a requirement for non-essential N in a low-protein diet rather than a requirement for glycine *per se* (Snyderman *et al.*, 1962).

**Table 1.1.**

*Classification of essentiality of amino acids in the human infant*

| Essential | Conditionally Essential | Non-Essential | Essentiality Undetermined |
|---|---|---|---|
| Isoleucine | Cysteine | Alanine | Arginine |
| Leucine | Histidine | Aspartic Acid | Glycine |
| Lysine | Tyrosine | Glutamic Acid | Taurine |
| Methionine | | Proline | |
| Phenylalanine | | Serine | |
| Threonine | | | |
| Tryptophan | | | |
| Valine | | | |

A summary of the classification of amino acids required by infants is given in Table 1.1. Although eight amino acids are still considered essential, there is enough evidence to include other amino acids in the category of conditionally essential. Given the relatively low activity reported for some of the enzymes involved in amino acid metabolism in the infant, there is the possibility that the infant's amino acid metabolism, in general, may have limited capacity. As such, it may be more appropriate to consider all amino acids as essential in the infant until evidence suggests otherwise, rather than add to the list of essential or conditionally essential amino acids as more information becomes available.

Having outlined which amino acids are required by the infant, determining the quantity of each amino acid required to achieve an acceptable rate of growth and well-being in the infant will now be discussed.

## 1.3 DETERMINING THE PROTEIN REQUIREMENTS OF THE INFANT

Prior to discussing the methods used to determine the protein requirement of the human infant, it is important to distinguish between requirements and recommended daily allowances (RDAs). The requirement is a physiological concept; the RDA is a generalisation based on the physiological conclusion and represents an application of requirements to a population (Waterlow, 1988). The RDA must therefore take account of the variability between individuals and is often set as the average requirement of a group plus two standard deviations, a so-called "safe level", in order to cover the needs of virtually all members of the group (Waterlow, 1988).

Although this approach may be valid for older children and adults, its application can be questioned with regard to breast-fed infants. Assuming that the average intake of protein in a population of breast-fed infants represents an average requirement for protein, it is illogical to suggest that to meet the requirement of the entire group, an allowance should be recommended that in most cases is greater than the actual intake. Based on this argument Waterlow (1990) concluded that RDAs have no meaning for the infant that is exclusively breast-fed. The following discussion, therefore, pertains to the estimation of an infant's protein requirement.

The requirement for protein during infancy can either be determined based on empirical observations (ie factorial approach, N balance or isotope tracer studies) or be based on epidemiological observations. Estimates of the protein requirement of an infant, determined using several different methods, are summarised in Table 1.2.

There is a large variation in the estimates of the protein requirements for infants. This arises, not only from the different techniques used, but also the philosophy behind the applications of the techniques. Differences in the objectives for determining the protein requirements of the infant are mainly focused on what criteria are to be achieved. The argument as to whether minimum or optimal requirements should be determined appears to be unresolved.

**Table 1.2.**

*The protein requirement of infants aged 3 months*

| Method of Determination | mg/kg/day | Reference |
|---|---|---|
| *Factorial Estimation* | 1.8 | FAO/WHO/UNU (1985) |
| | 1.4 | Fomon (1986) |
| | 1.4 | Butte & Garza (1985) |
| | 1.5 | Fomon (1991) |
| *N Balance/Intake Data* | 1.7 | Fomon *et al.* (1973) |
| | 2.0 | Fomon *et al.* (1971a) |
| | 2.0 | FAO/WHO (1971) |
| *Human Milk Intake* | 1.7 | FAO/UNU/WHO (1985) |
| | 1.2 | Fomon (1991) |
| | 1.5 | Janas *et al.* (1985) |
| | 1.1 | Butte *et al.* (1984a) |
| | 1.4 | Butte & Garza (1985) |
| | 1.3 | Fomon (1986) |

### 1.3.1 Factorial Method

The factorial approach has been used to estimate the mean protein requirement of infants (Hegsted, 1964; Food and Agriculture Organisation/World

Health Organisation[2], 1973; El Lozy and Hegsted, 1975) and consists of summation of the protein requirements for growth and for replacement of obligatory losses in urine and faeces and from the skin. Although replacement of losses accounts for the entire protein requirement of adults, and all but a small proportion of the requirement of children older than 2 years of age, accretion of protein accounts for a much greater proportion of the requirement of the young, rapidly growing infant (Fomon, 1991). The assumptions used to estimate the different avenues of N loss or accretion are critical in accurately determining the protein requirement factorially.

The obligatory loss of N from the body is usually determined under conditions of protein-free alimentation. For obvious ethical reasons, few studies have investigated the obligatory loss of N in infants. In the past, the assumption has been made that the infant's maintenance N requirement could be calculated from estimates of obligatory N loss in adults (El Lozy and Hegsted, 1975). Studies of N metabolism and protein turnover in infants (Pencharz *et al.*, 1977), however, suggest that tissue protein maintenance represents a more active metabolic state in the neonate. This is supported by evidence from Fomon and colleagues. They conducted a series of metabolic studies with infants, from birth to 6 months of age, fed human milk from a bottle (Fomon and May, 1958) and with infants from 4-6 months of age fed diets extremely low in protein (Fomon *et al.*, 1965). From these studies, they estimated that the inevitable loss of N by infants was 141 mg N/kg/day which is more than twice the 54 mg of N/kg/day reported by Calloway and Margen (1971) to be necessary for the maintenance of N balance in the adult. The obligatory N losses in infants were not determined under protein-free dietary conditions, which may explain in part the higher loss rate. This may not be a limitation in the measurement, however, for as discussed by Oddoye and Margen (1979), measuring N status while subjects are actually consuming protein seems more logical than measuring N loss in an individual fed a protein-free diet.

The protein required for growth consists of two components; the deposition of new tissue, as reflected in weight gain, and an increase in the N concentration in the body which is often referred to as maturation (FAO/WHO, 1973). Based on the measurement of body water, Fomon *et al.* (1982) estimated the accretion of N at different ages. The greatest rate of increase in body N is in the first year of life, mature composition being attained by the fourth year of age. Therefore estimation

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[2] *Hereafter FAO/WHO (1973)*

of the protein required by the infant for growth needs to take into consideration both the growth and maturation of body tissues. Estimating the protein required for accretion is difficult, however, due to limited information being available on the efficiency of conversion of dietary protein to body protein.

Dietary amino acids are not used with 100% efficiency within the body (Chung and Baker, 1992), and estimation of the efficiency of utilisation of dietary protein needs to be included in the factorial procedure before a definitive recommendation on the requirement for protein can be made. In the 1985 Food and Agriculture Organisation/World Health Organisation/United Nations University[3] report, the lowest estimates of the N required to obtain equilibrium in adults or satisfactory growth in children were consistently higher than the predictions of N required based on the factorial method. They predicted that dietary protein, even high quality protein, was only utilised with a 70% efficiency. Fomon (1991), however, predicted that in infants the efficiency of conversion was close to 90%. The lack of understanding with regard to the efficiency of utilisation of dietary protein and the assumptions required with the factorial method has tended to discourage its use (FAO/WHO/UNU, 1985; NRC, 1989), with the regulatory groups favouring recommendations on protein requirements based on N balance studies.

### 1.3.2 Nitrogen Balance Studies

An overall assessment of protein status in the body can be made using the N balance technique. This involves determining the difference between the daily intake of N and the daily excretion of N in the form of urine, faeces and sweat together with minor losses occurring by other routes (FAO/WHO/UNU, 1985). Nitrogen balance response curves are derived by giving an individual a series of different amounts of dietary protein and determining the balance of N at each of these different amounts. The requirement is estimated by extrapolation or interpolation of the N balance data to the zero balance point for adults (maintenance requirement) or to a defined level of positive balance for children (maintenance and growth requirement). This technique has been used to determine the protein requirements of infants, children and adults (FAO/WHO/UNU, 1985). The N balance concept has also been used to identify which amino acids were essential in adults (Rose, 1957) and infants (Holt and Snyderman, 1965) based on the physiological phenomenon that N balance (either to

[3] *Hereafter FAO/WHO/UNU (1985)*

zero or some specified level) will not be obtained or maintained when any dietary essential amino acid is ingested in insufficient amounts.

There are two main concerns, however, about the use of N balance studies to determine protein requirements for infants. Firstly, controversy exists as to the level of growth and the associated positive N balance deemed to be satisfactory in infants. Secondly, the N balance method has several inherent limitations.

Estimates of protein requirements in infants determined in N balance studies (Fomon and May, 1958; Fomon, 1961; Holt and Snyderman, 1965; Fomon *et al.*, 1973) have relied on the use of a standard growth pattern. The growth parameters used were derived from formula-fed infants. Historically, formula-fed infants had higher growth rates than breast-fed infants (Whitehead and Paul, 1981), although there has been a general reduction in the differences as formulas have been modified in an attempt to match the composition of human milk (Casey, 1989). In general, however, estimates of the infant's requirement for protein, determined using this latter method were considerably higher than the average protein intake of breast-fed infants. This leads to the question as to which criterion for growth is appropriate, ie normal (breast-fed infants) versus maximal (formula-fed infants).

Besides the issue of selecting a satisfactory level of positive N balance, the method has several inherent limitations and the results obtained need to be interpreted accordingly. As the concept of N balance and the limitations of this method have been extensively reviewed (Holmes, 1965; Hegsted, 1976; Rand *et al.*, 1981; Manatt and Garcia, 1992) only a summary of the major limitations will be given in this review.

With particular reference to the use of N balance methods to determine a human's requirements for individual amino acids, the suitability of synthetic and semi-synthetic diets as opposed to protein-based diets in determining N balance has also been discussed (Millward *et al.*, 1989). The omission of and supplementation with specific amino acids can give rise to unknown interrelationships between amino acids (Millward and Rivers, 1988). One such interrelationship, reported by Holt and Snyderman (1961), demonstrated that when isoleucine was omitted from the diet pronounced abnormalities developed in the amino acid pattern of the blood serum of infants, in particular there was a dramatic increase in the serum tyrosine level.

Nitrogen balance studies, particularly with infants and children, are often conducted in metabolism wards in hospital, and usually involve the restraint of the

infant in a metabolic frame for up to 72 hours. There is the concern that data collected from subjects living in a controlled metabolic ward may not be representative of normal living conditions (Scrimshaw, 1977; Jackson, 1983). Although there is the possibility that they may induce an abnormal N metabolism, the use of metabolic wards and frames allows the careful monitoring of N intake and excretion (Irwin and Hegsted, 1971). Despite careful attention to detail, there is still the potential in any N balance study for systematic overestimation of retention indicated by unrealistic positive balances and low estimated requirements (Calloway and Margen, 1971; Hegsted, 1976). Also, because the resultant balance figure is a small number derived from two much larger numbers, any isolated or systematic sample collection or analytical error can significantly influence the final result (Manatt and Garcia, 1992).

Although the N balance technique has well documented limitations, it remains a useful tool in the determination of protein and amino acid requirements in infants, assuming that a suitable point of positive balance can be agreed upon. The concept of N balance, however, does not necessarily represent adequate protein nutrition, in part because the N balance method does not reveal information about the distribution of body N among tissues and organs (Young, 1987), and also because the existence of reserve or labile protein in the body or the previous protein status in the body may influence the N balance calculation (Waterlow, 1968). In addition, adaptation and accommodation of N metabolism with varying levels of dietary protein (Millward *et al.*, 1989) raises doubt with regard to the N balance method. It is possible, however, through the use of isotopically labelled amino acid tracers, to investigate the metabolism of N within the body towards determining amino acid requirements.

### 1.3.3 Isotope Tracer Methods

A major criticism of the N balance method for determining the protein requirements of adults is that adaptation and accommodation of N metabolism can occur when the intake of protein is reduced as would be the case in the determination of obligatory N loss in adults (Young and Marchini, 1990). This is evidenced by the significant reduction in the rate of body protein turnover in healthy adults given intakes of leucine approximately equal to or less than the currently recommended requirement (Young *et al.*, 1987). Whereas the N balance method provides information about the net changes in body N content, measuring the kinetics of N

metabolism will give a much better appreciation of the metabolic pathways involved in N metabolism. This in turn should lead to more reliable estimates of the requirement for protein and essential amino acids in humans.

The kinetic model assumes that dietary amino acids have two functions, a substrate role for protein synthesis and all other metabolic transformations, and a regulatory influence on many of the individual pathways involved in their utilization. This latter function is often termed the anabolic drive of amino acids and can be defined as the transient regulatory influence dietary essential amino acids have on growth and protein turnover before they are oxidised (Millward and Rivers, 1988). Thus measuring the regulation of whole-body protein turnover provides a better understanding of the requirement for individual amino acids.

The many approaches to studying the kinetics of protein metabolism using stable isotopes have been extensively reviewed (Picou and Taylor-Roberts, 1969; Zak *et al.*, 1979; Waterlow *et al.*, 1978; Young and Bier, 1987; Hoerr *et al.*, 1989; Young, 1989; Schreurs *et al.*, 1992). Amino acid tracers (amino acids labelled with either $^{15}N$, $^{13}C$ or in some cases $^{2}H$) are administered into the body either orally or intravenously, and in either a single bolus or by continuous infusion. There are two main approaches to using isotopically labelled amino acid tracers.

The first involves the stable isotope $^{15}N$. By various N exchange processes, it is assumed that $^{15}N$ is released from the amino acid tracer and homogenously mixed throughout the body. After an appropriate delay to allow complete distribution, the N pool is sampled to measure dilution of the tracer. It is assumed that this dilution has occurred by dietary N intake and by unlabelled N release from protein breakdown. The rate of protein turnover is therefore determined based on the rate of dilution. As it is not possible to sample the N pool directly, the $^{15}N$ in the end-products of N metabolism (ie. urea and ammonia) are measured. This method relies on $^{15}N$ leaving the administered amino acid tracer and mixing throughout what is believed to be a single N pool.

In an alternative approach, a $^{13}C$-labelled amino acid acts as a representative tracer for body protein amino acids. The enrichment of this amino acid tracer in the plasma is measured, and whole-body protein turnover is calculated from an assumed average content of this amino acid in body protein. This method, which is quicker than the end-products method and therefore probably more applicable in clinical studies, relies on the stable isotope label remaining in the infused amino acid tracer

or being irreversibly lost through oxidation. The latter process can be monitored by measuring the dilution of $^{13}C$ exhaled in the breath.

There are many assumptions and limitations associated with the use of stable isotopes and these have been well documented (Bier and Young, 1986; Millward and Rivers, 1988; Young *et al.*, 1988; Millward *et al.*, 1991). Associated with the end-products method are the assumptions that there is a single metabolic N pool, the tracer is uniformly distributed, the tracer is not diluted significantly by N from sources other than the diet or protein N, and that the proportion of tracer N to total N entering the end-products is equal to the fraction entering protein. Many of these assumptions remain unproven or are incorrect leaving the validity of $^{15}N$ studies in doubt (Bier and Young, 1986). Although the representative method does not require as many assumptions as the end-products method, it does rely completely on the assumption that the label remains in the infused tracer or is lost through oxidation. In addition, there are practical problems associated with this method including the logistics of measuring exhaled $CO_2$ and the amount of tracer needed, which may negate the assumption of a true tracer. Losses of tracer via bicarbonate excretion in the gut have been reported. Furthermore, there is inadequate understanding of the relationship between the isotopic abundance in the measured substance (ie plasma amino acid) and the actual precursor for the process under study (protein synthesis or oxidation, or both). Despite these limitations, Millward *et al.* (1991) concluded that stable isotope tracer methods in the study of whole body protein turnover could be used with relative confidence.

A series of studies using stable-isotope tracers have been conducted to reassess the protein requirements of adult man (Meguid *et al.*, 1986a; Meguid *et al.*, 1986b; Meredith *et al.*, 1986; Zhao *et al.*, 1986; Young *et al.*, 1987; Cortiella *et al.*, 1988; Pelletier *et al.*, 1991a; Pelletier, 1991b). The conclusion drawn from these studies was that the "true" requirements for at least the four amino acids (leucine, threonine, valine and lysine) tested were approximately two to three times higher than the currently accepted FAO/WHO/UNU (1985) values and the difference may represent the anabolic drive function of amino acids in addition to their contribution to N balance.

The rate of protein turnover in infants has been estimated using tracer methods (Nicholson, 1970; Pencharz *et al.*, 1977; Frazer and Bier, 1980; Jackson *et al.*, 1981; Heine *et al.*, 1983; Stack *et al.*, 1989; Kandil *et al.*, 1991). Most of the studies, however, have been conducted with low-birth weight or preterm infants, with only

one study investigating protein turnover in newborn full-term infants (Frazer and Bier, 1980). The rate of protein synthesis determined in the infants in these studies ranged from 5 to 26 g protein synthesis per kg bodyweight per day. Although the majority of the studies used the end-products method, the dosage rate, methods and times of sampling varied considerably, which may explain the large range of estimates. In particular, the investigation of protein turnover in breast-fed infants using the end-products method was impeded by the high concentration of un-labelled urea in human milk (Pencharz *et al.*, 1989) which influenced the dilution of the tracer in the infant's metabolic N pool.

As none of the experimental factors on which the calculation of protein turnover depends have been rigorously tested in the human infant, and given the number of assumptions and limitations surrounding the stable isotope methods, Bier and Young (1986) concluded that whole-body protein dynamics measured in infants should be interpreted with caution. Indeed, Millward and Rivers (1988) suggested that because the criterion for N balance in infants dictates a positive balance, rather than equilibrium as with the adult, there is less likelihood of adaptation and accommodation of the infant's N metabolism. They added that allowing for growth of the infant in the N balance method also accounted for the anabolic drive of amino acids. As such they believe that there is less concern with regard to the infant's protein requirements determined using the N balance method. Although this may be correct, with increased use and availability of stable isotope technology, the potential now exists to quantify more precisely the various aspects of amino acid metabolism in the neonate.

### 1.3.4 Epidemiological approach

One further approach to determining an infant's protein and amino acid requirements is to measure the protein intake of a population of infants believed to be healthy and growing at a satisfactory rate, and this technique has been applied extensively (Fomon and May, 1958; Fomon *et al.*, 1969; Filer *et al.*, 1970; Fomon *et al.*, 1971a; Fomon *et al.*, 1971b; Fomon *et al.*, 1973; Räihä *et al.*, 1976; Järvenpää *et al.*, 1982a; Whyte *et al.*, 1983). Although this may seem the simplest and most appropriate method for determining amino acid requirements for infants, there are several aspects of this approach that need to be considered. Firstly, there is the argument that the average intake of a population cannot, by definition, represent the requirement

because in doing so 50% of the population would automatically be receiving less than required (Waterlow, 1990).

Beaton and Chery (1988) discussed in depth, application of the epidemiological approach to estimate the protein requirements of infants, with particular reference to the difference between average intakes and average requirements. They argued that if the correlation between intake and requirement in a population of breast-fed infants was 0.3, 30% of the population would be receiving less protein than they required, which based on clinical growth studies, seems unlikely. Alternatively, they suggest that the average N requirement is substantially less than the average intake. From this they estimated by statistical prediction, a protein requirement considerably lower than the protein intake of breast-fed infants. Their calculations depend on a number of assumptions, however, of which there is very little independent support (Waterlow, 1990).

The second issue of concern with the epidemiological approach, is the criterion used to define healthy, growing infants. This has already been discussed to some extent and can be summarised as a conflict between what is considered normal and what is considered optimal in terms of infant growth.

In the report by the FAO/WHO/UNU (1985), one estimate for the protein requirement of infants was derived from the average intake of protein in a population of formula-fed infants. The average intake of protein by breast-fed infants (Whitehead and Paul, 1981; Janas *et al.*, 1985; Neville *et al.*, 1988) is lower than the FAO/WHO/UNU (1985) recommended protein requirement which implies that breast-fed infants are not receiving enough protein to meet their requirements. This is obviously not correct, although breast-fed infants do grow at a slower rate than formula-fed infants (Whitehead and Paul, 1981). Many believe that the lower growth rates observed in breast-fed infants should be considered normal and used as the standard when assessing the infant's requirements for protein and amino acids (Whitehead and Paul, 1981; Salmenperä *et al.*, 1985; Räihä *et al.*, 1986a). Fomon (1986) argues, however, that since formula-fed infants gain more lean body mass than breast-fed infants, breast-feeding is not an optimum, but a compromise which allows for adequate growth of the infant without undue depletion of maternal body protein. It would appear more logical, however, to consider the intake of protein by breast-fed infants as the infant's optimal requirement for protein and amino acids.

When determining the intake of protein by breast-fed infants one further detail

needs to be considered. The protein content of human milk is often determined by multiplying the total N by a conversion factor, usually 6.38 (FAO/WHO/UNU, 1985). This assumes that all of the N in human milk is in protein form and therefore represents the infant's protein requirement. Non-protein N accounts for a major fraction of the N in human milk, averaging approximately 24% of the total N in mature milk (Macy, 1949; Lönnerdal *et al.*, 1976b; Harzer *et al.*, 1984). Urea N comprises as much as 50% of this non-protein N, or up to 10% of the total N (Hambraeus *et al.*, 1978). Differences in metabolism between breast-fed and formula-fed infants, summarised by Butte and Garza (1985), suggest that because efficiency of N utilisation is dependent on protein intake, breast-fed infants, whose intakes are likely to be closer to the true requirement, may conserve more N relative to formula-fed infants by utilising some portion of the non-protein N available to them.

A number of studies have investigated the extent to which dietary urea is utilised in the infant. Snyderman *et al.* (1962) demonstrated that weight gain could be restored in infants fed low-protein diets by the administration of nonessential N in the form of glycine or urea. Dietary urea can only be utilised by humans after it has been degraded by intestinal bacterial ureases with subsequent absorption of ammonia and the use of this ammonia for amino acid synthesis. Urease-containing bacteria such as the *Bifidobacterium* species and *Escherichia coli* are found in the normal colonic flora of both breast-fed and formula-fed infants (Heine *et al.*, 1984).

In several studies $^{15}N$ labelled urea has been used to measure the incorporation of $^{15}N$ into whole body protein in infants (Heine *et al.*, 1986; Heine *et al.*, 1987; Fomon *et al.*, 1987; Fomon *et al.*, 1988; Donovan *et al.*, 1990). The degree of $^{15}N$ retention ranges from 13% to 43%. The higher levels of $^{15}N$ retention were observed in infants that were compromised, for example low-birth-weight infants, or those recovering from either enteritis or infections of the respiratory passage or urinary tract. In contrast, the infants studied by Fomon *et al.* (1987, 1988), having among the lowest $^{15}N$ retention, were normal, full-term infants between two and ten months of age receiving what was believed to be adequate protein prior to and during the study either in the form of human milk (Fomon *et al.*, 1988) or infant formula (Fomon *et al.*, 1987). Furthermore, Rose and Dekker (1962) demonstrated that the utilisation of dietary urea in rats was dependent upon the protein content of the diet with the incorporation of labelled urea into whole-body protein being significantly reduced (from 12% to 0.4%) when dietary N was adequate.

It would be expected, therefore, that a healthy breast-fed or formula-fed infant receiving an intake of protein close to or in excess of requirements is not likely to rely on dietary urea to supplement protein synthesis. From this it can be concluded that most of the urea in human milk is not available to the infant for nutritional purposes.

Therefore, multiplying the total N in human milk by 6.38 to determine the protein content is incorrect, and will lead to an overestimation of the infant's protein requirements. The true protein content of human milk can be more accurately determined by analysis of the amino acid composition of the human milk, and from this the amino acid requirements of the infant can be determined.

**Table 1.3.**

*Different estimates of the amino acid requirements* of human infants aged 0-6 months*

| Amino Acid | Method of Determination | | | |
|---|---|---|---|---|
| | Nitrogen Balance+ | Formula Intake# | Breast Milk Intake§ | Breast Milk Intake¶ |
| Histidine | 34 | 28 | 39 | 34 |
| Isoleucine | 119 | 70 | 70 | 101 |
| Leucine | 150 | 161 | 140 | 153 |
| Lysine | 103 | 161 | 100 | 95 |
| Methionine | 45 | 58 | 64 | 19✿ |
| Phenylalanine | 90 | 125 | 109 | 98 |
| Threonine | 87 | 116 | 65 | 65 |
| Tryptophan | 22 | 17 | 26 | -= |
| Valine | 105 | 93 | 83 | 67 |

* mg of amino acid/kg body weight/day.
\+ Holt and Snyderman (1965).
\# Fomon and Filer (1967).
§ FAO/UNU/WHO (1985): Human milk amino acid pattern mg/g crude protein (total N x 6.25) multiplied by an expected intake of 1.51 g protein/kg/day in 3 month old infants.
¶ Janas and Picciano (1986).
✿ Methionine only.
= Not determined.

### 1.3.5 Summary

Due to the variety of approaches used to determine the amino acid requirements of human infants, there are considerable differences in the final estimates. A range of estimates for the amino acid requirements of infants aged between birth and six months of age are given in Table 1.3. Although most of the methods attempt to investigate the physiological basis for the requirement they are not without limitations. By far the simplest and perhaps most logical approach is to determine the infant's requirements based on the average intake of amino acids by healthy breast-fed infants assuming that all the amino acids in human milk are completely absorbed.

## 1.4 DETERMINING THE AMINO ACID REQUIREMENTS OF INFANTS BASED ON THE GROSS AMINO ACID COMPOSITION OF HUMAN MILK

If the average intake of amino acids by healthy breast-fed infants is to be used as the basis for determining amino acid requirements for infants, it is important to considered the physiological function of breast milk, and its adequacy in fulfilling that role. The volume of milk produced by the mother in relation to the infant's requirements also needs to be investigated. Whether the samples of milk analysed are representative of the milk produced by a population of women will also influence the final determination of requirements as will the methods of collection and the accuracy of amino acid analysis. The following discussion examines each of these issues.

### 1.4.1 The role of milk

The essential characteristic of mammals is the capacity of the female to nourish her offspring with milk, the secretion of the mammary glands (Neville and Neifret, 1983). Jenness and Sloan (1970) described milk as a balanced multi-component dietary system with components having separate as well as partially overlapping functions. Milk provides nutrition for the young mammal, supports the growth of symbiotic intestinal flora and transmits passive immunity.

The composition of milk changes dramatically over the initial stages of lactation. The residual mixture of material present in the mammary glands and ducts at parturition and immediately after is progressively diluted with newly secreted milk forming the 'first-milk' or colostrum, and later the transitional milk. The colostrum

secreted during the initial 5 or 6 days after parturition is considered to have specific value and immunological properties for the newborn, who must clear its alimentary tract of meconium in preparation for the normal functioning of its own digestive, respiratory and urinary systems (Macy and Kelly, 1961). After the first week of lactation the milk changes from colostrum to transitional milk and finally to mature milk, that is, milk that has reached a relatively consistent composition.

In some species, milk is the sole food consumed for weeks or months, and as such, must meet all the neonate's nutritive requirements (energy, amino acids, minerals, and vitamins) for maintenance and growth (Jenness, 1985). In other species, the neonate begins to consume foods other than milk soon after birth, making the composition of the milk less critical in supplying the nutrients required by the neonate (Jenness, 1985). Animals such as ruminants, rely almost exclusively on milk to transfer antibodies from mother to young. Others, such as primates, transfer antibodies via the placenta and are not dependent on milk for this function. Given the varying physiological importance of milk in different mammalian species, there exists large interspecies differences in the quantitative composition of milk (Jenness and Sloan, 1970; Oftedal, 1984). As a consequence, human milk is generally considered as the best food for human infants during the first few months of life (Committee on Nutrition, 1982; Hartmann *et al.*, 1984; FAO/WHO/UNU, 1985; Casey, 1989; Dualeh and Henry, 1989) and there are several comprehensive reviews detailing the unique compositional characteristics of human milk (Hambraeus, 1982; George and DeFrancesca, 1989; Casey, 1989).

### 1.4.2 The adequacy of breast milk

Given that there is a large variation in the composition of milk from different mammalian species, it would seem reasonable to assume that through the process of evolution, the compositions of the milks have evolved to meet the specific nutrient requirements of the young of each species. Also, at peak lactation, it would appear to be a disadvantage to the mother to provide nutrients in excess of the neonate's requirements (Oftedal, 1986). Likewise, considering the high relative growth rates observed during infancy (Whitehead and Paul, 1981, Oftedal, 1984), and the obvious vulnerability of the young to nutrient deficiencies, it is unlikely that under normal conditions mammalian milks are limiting in essential nutrients. However, as mentioned previously, Fomon (1986) argues that on an evolutionary basis lactational

output is a compromise between the infant's requirements and the depletion of maternal protein stores, and as such probably does not represent the optimum nutrient intake.

The amino acid profile of human milk is compatible with the infant's metabolic capacity to synthesise and deaminate amino acids. Hartmann *et al.* (1984) noted that human milk contains only small quantities of tyrosine and phenylalanine which match the limited capacity of the infant's relatively immature liver to metabolise these amino acids. The high level of cysteine in human milk, compared to other milks such as bovine milk (Jenness and Sloan, 1970) may also be uniquely suited to the infant's supposedly limited conversion of methionine to cysteine.

Moreover, the intake of excessive amounts of amino acids by infants may have harmful effects on the infant's renal system (Berg *et al.*, 1987), or central nervous system and brain development (Goldman *et al.*, 1974). These findings all indicate that a narrow margin may exist between sufficient and excessive amino acid intakes in infants, and that human milk provides appropriate amounts of each amino acid.

However, human milk may not always be the most appropriate food for infants. In particular, controversy exists with regard to the feeding of mature human milk, often from milk-banks, to premature infants. While providing theoretically beneficial non-nutritional factors, mature human milk may be quantitatively deficient in protein, calcium, sodium, zinc and possibly other nutrients needed by the premature infant. Clinical studies of premature infants fed mature breast milk generally indicate that mature human milk does not provide optimal nutritional support to the premature infant (Räihä *et al.*, 1976; Davies, 1977; Munro *et al.*, 1988). The ability of the human milk secretion to adapt to the needs of the premature infant have been demonstrated, however, with the composition of milk from mothers delivering prematurely containing a higher concentration of protein nitrogen (Atkinson *et al.*, 1978; Gross *et al.*, 1980; Lemons *et al.*, 1982), sodium (Gross *et al.*, 1980; Lemons *et al.*, 1982), and other minerals (Lemons *et al.*, 1982).

In a review of the adequacy of breast milk, Coveney (1985) described how breast-feeding may be contraindicated in certain metabolic conditions such as galactosaemia and lactose intolerance in the infant. Coveney (1985) noted that the composition of breast milk, particularly the fat, mineral and vitamin content, is influenced by the maternal diet. Therefore, malnourished mothers may produce milk that is inadequate in meeting the infant's requirements for these nutrients. In

addition, nutrients such as vitamin K and biotin are present in very low amounts in human milk, the infant relying on its own gut flora to synthesise these nutrients. In this case, the level of these nutrients in human milk do not reflect the infant's requirement.

In general, however, breast milk can be considered appropriate for healthy full-term infants and as such is widely accepted as reflecting the nutritional requirements of infants.

### 1.4.3 Measurement of milk intake by the breast-fed infant

There is a wide range in estimates of milk intakes among normal, healthy, exclusively breast-fed infants. Intakes range from 450-1200 g/day with the average reported intake being between 700-800 g/day in the first 4 months (Wallgren, 1945; Lönnerdal *et al.*, 1976a; Hofvander *et al.*, 1982; Whitehead and Paul, 1981; Dewey and Lönnerdal, 1983; Butte *et al.*, 1984a). The criteria for, and the difficulty in, determining intakes in the infant, however, may contribute in part to the wide variation observed. It is possible to measure either the milk yield of the mother or the amount of breast milk consumed by the infant. Milk yield can be assessed by measurement of the volume of breast milk expressed from the breast by either manual expression or by the use of a breast pump. There is evidence to suggest, however, that direct measurement of the amount of milk expressed may be inaccurate due to interference with the processes of milk let down (Jelliffe and Jelliffe, 1978; Coward *et al.*, 1979). Furthermore, measuring milk yield using any of these methods gives an estimation of the milk synthesising capacity of the mother rather than an indication of the infant's intake.

It would seem more logical to measure the actual intake of the infant and the different techniques available for determining the intake of breast milk by infants have been reviewed (Coward, 1984; Hartmann *et al.*, 1985; Woolridge *et al.*, 1985). The most commonly adopted approach is to test-weigh either the mother or infant prior to and after a breast-feeding, taking the difference in weight as the amount of milk consumed (Arthur *et al.*, 1987). Movement of the infant during test-weighing significantly limits the accuracy of this method, although integration electronic balances that tolerate movement can be used. In addition, the method is limited by the need to correct for evaporative water loss which may not be significant in the infant, but may lead to an overestimation of intake when the mother is weighed

(Butte *et al.*, 1983; Arthur *et al.*, 1987; lmong *et al.*, 1988). An alternative technique involves enrichment of either the infant or the mother with deuterium oxide. Dilution of the isotope in the infant is monitored and assumed to be due to milk intake (Coward *et al.*, 1979; Butte *et al.*, 1983; Butte *et al.*, 1988). This method also has logistical limitations and can only provide an estimate of intake on a week by week basis rather than the daily intake.

Measurement of milk synthesis whereby breast volume is calculated from photographs of contour lines projected onto the breast using the technique of Moiré topography has been establised (Arthur *et al.*, 1989). This concept has been improved with video imaging and computerised integration (Daly *et al.*, 1992) to enable rapid measurement of a greater range of breast shapes and sizes. While designed to measure the rate of milk synthesis in the mammary gland, both of these methods can be used to determine milk intake in breast-fed infants.

Assuming that the intake of milk by breast-fed infants can be accurately measured, it is important to distinquish whether the range of estimated breast-milk intakes is due to maternal supply or infant demand, particularly when the average intake is used to determine the amino acid requirements of the infant. The potential milk volume of healthy lactating mothers appears to exceed what is consumed by the infant. This is demonstrated quite clearly by the ability of mothers of twins to produce twice the milk volume of mothers of singletons (Hartmann and Prosser, 1984). The greater the frequency, and the more completely the breasts are emptied at each breast-feeding, the more milk the mother appears to produce. It is widely accepted, therefore, that the wide range in breast milk volume in well nourished populations is due more to variation in infant demand than to inadequacy of milk production (Illingworth and Stone, 1952; Egli *et al.*, 1961; Hofvander *et al.*, 1982; Butte *et al.*, 1984a; Dewey and Lönnerdal, 1986).

Although there is a wide range in breast milk consumption in infants, within any given age group of infants, the intakes appear to be very similar on a body weight basis (FAO/WHO/UNU, 1985; Forsum and Sadurskis, 1986; Michaelsen *et al.*, 1994).

### 1.4.4 Definition of a representative milk sample

When determining the amino acid requirements of the infant based on the amino acid composition of human milk, it is important to ensure that the human milk

sample analysed is representative of human milk from a population of healthy, well-nourished lactating women. Although the composition of human milk will change throughout the lactational period and can be influenced by a number of different factors, in general the protein content of human milk in a population of women is uniform in composition at any given stage of lactation (Hall, 1979; Picciano, 1984). Those factors that may influence the protein composition of human milk are now discussed.

#### *1.4.4.1 Stage of Lactation*

The changes that occur in the composition of human milk during lactation have been extensively researched (Morrison, 1952; Hytten, 1954a; Lönnerdal *et al.*, 1976b; Harvey Anderson *et al.*, 1981; Anderson *et al.*, 1983; Butte *et al.*, 1984b; Lewis-Jones *et al.*, 1985a; Ferris *et al.*, 1988; Allen *et al.*, 1991; Michaelsen *et al.*, 1994). Concentrations of protein are highest in colostrum then decline significantly during the first few weeks of lactation reaching relatively stable concentrations from day 15 of lactation onwards. The latter milk secretion is often referred to as mature human milk. The protein content has been found to increase again at weaning (Dewey and Lönnerdal, 1984; Neville *et al.*, 1986). Therefore, the mean protein content of human milk decreases from approximately 2g/100ml on day 2 to about 1g/100ml in mature milk. Accordingly, exclusively breast-fed infants have a protein intake that decreases from about 2g/kg/day during the first month to about 1g/kg/day from 4 to 6 months of age.

The majority of studies that have investigated the changes occurring in the protein content of human milk during lactation have only measured total N. The individual proteins in human milk vary in the rate at which they decline during the initial stages of lactation (Kulski and Hartmann, 1981; Lewis-Jones *et al.*, 1985a; Harzer and Bindels, 1987), with a sharp decrease in the secretory immunoglobulin A (sIgA) content and a moderate decrease in the lactoferrin content. Casein, alpha-lactalbumin, albumin and lysozyme appeared to remain constant. Changes in milk protein concentrations will have a significant effect on the concentrations of individual amino acids in human milk because the different protein fractions have different amino acid profiles (Harzer and Bindels, 1987). This has direct implications with regard to determining the amino acid requirements of the infant based on the amino acid composition of human milk. The stage of lactation at which the representative milk sample is to be taken will directly influence the amino acid profile.

*1.4.4.2 Variations during a single breast-feeding, and during the day*

The milk secreted during a single breast-feeding can be seen to change in consistency and colour, reflecting a changing composition. In the most part, this change is limited to an increase in the concentration of fat (Hytten, 1954b). Although Hall (1979) reported slight increases in protein content during a feed, most research suggests that there is no significant change in the concentration of total nitrogen in human milk during a feed (Macy *et al.*, 1931; Hytten, 1954b; Neville *et al.*, 1984). Hytten (1954b) did observe changes in the type of N during a feed, however, with casein, which may be partially adsorbed to the fat fraction, rising slightly at the end of a feed, whereas the soluble protein fraction decreased. He suggested that the pattern of amino acids in the milk during a feed would depend, therefore, on the relative proportions of the milk proteins.

There appears to be no circadian variation in the protein content of human milk (Hytten, 1954c; Lönnerdal *et al.*, 1976b; Lammi-Keefe *et al.*, 1990).

*1.4.4.3 Dietary Influences*

There have been several reviews on the effect of maternal diet on the composition of human milk (Hartmann *et al.*, 1985; Finley, 1986; Lönnerdal, 1986a). In general, it appears that the nutritional status of the mother has little, if any, effect on protein concentration. In a series of studies, reviewed by Lönnerdal (1986a), the protein concentration in milk collected from women in different countries, namely India, Pakistan , Ethiopia, Nigeria, Kenya and the Gambia, were compared with the protein content of human milk from well-nourished mothers in Sweden and Great Britian. The protein content of the mature human milk from all of these women was 0.8-1.0% regardless of maternal nutrition. Several studies have reported an increase in the concentration of protein in human milk when a severely malnourished mother is given a protein-supplemented diet (Forsum and Lönnerdal, 1980; Prentice *et al.*, 1983). The increases, however, were only in the region of 7-10% of total N, and may have reflected a change in the non-protein N rather than a change in the amino acid content of the milk.

In comparison to well-nourished lactating women, however, malnourished mothers appear to have higher proportions of lactoferrin (Lönnerdal *et al.*, 1976a), lysozyme (Miranda *et al.*, 1983), albumin (Miranda *et al.*, 1983) and the immunoglobulins (Prentice *et al.*, 1984) in their milk. De Ferrer and Sambucetti (1992)

also demonstrated that the relative proportions of milk proteins were altered when the protein in the diet was changed, with the whey protein:casein ratio being positively related to maternal protein intake. Thus, it is evident that although total protein content may not be affected by maternal diet, the amino acid composition may be, particularly in malnourished mothers.

#### *1.4.4.4 Other factors affecting composition*

Researchers have investigated the effects of maternal age and parity (Butte *et al.*, 1984c; Lewis-Jones *et al.*, 1985b), menstruation (Hartmann and Prosser, 1982), mastitis (Ramadan *et al.*, 1972), oral contraceptives (reviewed by Lönnerdal, 1986b), smoking (Vio *et al.*, 1991), exercise rates (Loveday *et al.*, 1990) and *diabetes mellitus* (Butte *et al.*, 1987) on the yield and composition of human milk. In most of these studies the factor investigated had no effect on the protein composition of mature human milk. The total nitrogen and the whey protein contents of breast milk appear to decrease and increase, respectively, if a breast infection is present. The effects of oral contraceptives on the protein content of human milk are contradictory. Lönnerdal (1986b) concluded that due to widely varying dosages and hormone combinations, and the stage of lactation at which they were administered to the women, the effects of oral contraceptives on the protein content of human milk was difficult to evaluate. In general, however, none of the factors discussed would appear to have a major bearing on the use of the amino acid composition of mature human milk to determine the amino acid requirements of human infants.

### 1.4.5 Accurate determination of the amino acid composition of human milk

With the knowledge that the protein composition is relatively stable in mature human milk and that most external factors, specifically the maternal diet, do not influence the protein content of the milk from well-nourished healthy lactating women, two further aspects of obtaining a representative human milk sample need to be considered. The method of milk collection and the conditions of storage may have a significant effect on the final amino acid composition. Finally, the method of amino acid analysis used will decide the accuracy with which the final amino acid composition of human milk is determined.

*1.4.5.1 Collection and storage procedures*

The human milk collected for analysis should be representative, both in quantity and composition, of that the infant would have suckled. There are several methods for collecting breast milk. The collection of milk that spontaneously drips from the contralateral breast during a feeding has been traditionally used to obtain milk for analysis. "Drip" milk, however, has a different composition to breast milk that has been manually or electrically expressed (Gibbs *et al.*, 1977; Hamosh *et al.*, 1984), and consequently this method is not recommended. Although collection of either the fore milk or hind milk, or both will cause little disruption to the suckled infant, the milk collected will not be representative of the entire breast secretion, particularly given the changes that may occur during a feed (Hytten, 1954a). It is preferable to collect the entire breast secretion as this will give the most accurate estimate of the nutrients received by the infant (Hamosh *et al.*, 1984). Although the breast milk can be expressed manually, more representative samples are obtained using an electric breast pump (Green *et al.*, 1982; Garza *et al.*, 1982; Hamosh *et al.*, 1984; Picciano, 1984). A less invasive method of collecting a representative sample of breast milk has been developed by Lucas *et al.* (1982) and involves the use of a nipple shield. The shield relies on negative pressure produced by the infant to extract a small sample of milk continuously during a feed. No more than 5% of the total intake is collected (Lucas *et al.*, 1982). Although this method causes the least disruption to the feeding process, fractional test weighing showed a tendency for a larger proportion of the feed to be collected at slower flow rates which in turn influenced the final composition of the collected sample (Jackson *et al.*, 1987). The recommended collection procedure entails the pooling of entire breast secretions, collected using an electric breast pump, from alternate breasts and at different times of the day, for each woman studied.

The methods used to handle and store the milk will also influence the composition of the milk sample. Due to the establishment and popularity of milk banks there has been considerable research in the area of handling and storage of milk samples (Department of Health and Social Security[4], 1981; Garza *et al.*, 1982; Goldblum *et al.*, 1982; Friend *et al.*, 1983; Berkow *et al.*, 1984; Hamosh *et al.*, 1984). Factors to consider in the storage of milk are the type of storage container used and the temperature at which the milk is stored. Adherence of sIgA and cellular

[4] *Hereafter DHSS (1981)*

components to material such as pyrex, polyethylene and polypropylene have been reported. The adherence appears to be time dependent, however, with less adherence after extended storage. While no significant loss of protein N has been observed in milk stored at 4°C for up to 48 hours (Hamosh *et al.*, 1984), extended storage at this temperature resulted in an increase in total N, presumably from bacterial growth (Garza *et al.*, 1982). Storage of milk at temperatures less than -20°C for up to 3 months has no effect on the milk enzymes. As three months is the longest interval recommended for storage of human milk in milk banks, the effect of longer storage times on the protein fraction of human milk has not been investigated.

### *1.4.5.2 Amino acid analysis*

To accurately determine the amino acid composition of a protein, four aspects of the methodology of amino acid analysis need to be considered. Namely, the preparation of the sample, the hydrolysis of the protein, the length of hydrolysis interval, and the method of amino acid detection. The optimal conditions of sample preparation and hydrolysis have been reviewed (Davies and Thomas, 1973; Blackburn, 1978; Finley, 1985; Zumwalt *et al.*, 1987; Food and Agriculture Organisation/World Health Organisation[5], 1990).

**Sample Preparation**: In most instances, the hydrated sample to be analysed is dried, preferably by freeze-drying, and finely ground to ensure an homogeneous mix. Samples with a high fat content, such as human milk need to be defatted prior to analysis as a fat content greater than 5% may interfere with the recovery of amino acids due to the reaction of oxidised lipids with the protein (Finley, 1985). Removal of carbohydrate from a sample prior to amino acid analysis, although recommended by Block and Weiss (1956), is not routinely carried out. A source of error frequently overlooked in amino acid analysis is contamination of glassware through handling (Finley, 1985). The simplest way to avoid contamination is to pyrolyze the glassware at 650°C before use. Careful sample preparation and handling of glassware will help ensure that a representative sample is ready for hydrolysis.

**Hydrolysis of proteins**: The hydrolytic step is possibly the greatest source of error in the determination of a protein's amino acid composition. Variations in the ease of peptide bond cleavage, differences in amino acid stabilities, and matrix effects from nonproteinaceous components all prevent the establishment of a single set of

[5]*Hereafter FAO/WHO (1990)*

hydrolysis conditions that will quantitatively hydrolyse every peptide bond and yet not destroy any amino acid. The use of different procedures or conditions for the hydrolysis of proteins has been extensively researched (Kohler and Palter, 1967; Davies and Thomas, 1973; Lucas and Sotelo, 1982; Gehrke *et al.*, 1985; Gehrke *et al.*, 1987; Zumwalt *et al.*, 1987). The procedure routinely adopted for hydrolysis of proteins involves the evacuation and sealing under vacuum of a tube containing a sample in 6 M HCl. Hydrolysis of a protein sample in a large volume of acid helps to minimise the loss of amino acids which can result from side-reactions with sample contaminants such as carbohydrates (Blackburn, 1978). In a review of several different studies into the effects of the acid:sample ratio on the final amino acid composition of a protein, Finley (1985) concluded that 1 mg of protein per ml of 6 M HCl was close to optimal. Re-distilled phenol should be added to the acid solution (1% w/w) to give a better recovery of both tyrosine and serine (Glazer *et al.*, 1976; Blackburn, 1978). The sealed tubes are usually incubated at 110°C for 24 hours. Variations on this procedure include an increase in the temperature in conjunction with a reduction in the length of hydrolysis (ie. 145°C for 4 hours) (Gehrke *et al.*, 1985), the use of different acids (Davies and Thomas, 1973), and different hydrolysis vessels such as screw-cap tubes versus heat-sealed ampules (Gehrke *et al.*, 1985).

Both cysteine and methionine are susceptible to partial oxidation during acid hydrolysis. To accurately determine these amino acids, cysteine and methionine can be quantitatively oxidised to methionine sulphone and cysteic acid using performic acid (Moore, 1963). The ratio of performic acid to protein is critical, however, with the optimum ratio being in the range of 0.08-1.3 ml performic acid/mg protein (FAO/WHO, 1990).

In the presence of cysteine or carbohydrate, tryptophan is completely destroyed during acid hydrolysis (Freidman and Finley, 1971), producing ammonia as the only recognisable end product. Alkaline hydrolysis of a protein does not destroy tryptophan, however, and it is the typical method for analysis. Critical factors in the alkaline hydrolysis of proteins are the sample to base ratio, choice of base, degassing procedure, time of hydrolysis, and the method of neutralisation (Finley, 1985). The sample size for alkaline hydrolysis of a protein should also be much larger than for other methods due to the lower concentration of tryptophan in most proteins.

**Hydrolysis intervals**: Hydrolysis intervals have often been selected for convenience, the most commonly used intervals being 18, 21, 22 or 24 hours (with 6

M HCl). The time necessary to hydrolyse the peptide bonds varies with the amino acid. Generally, the peptide bonds between the aliphatic amino acids (isoleucine, leucine and valine) are the most difficult to separate (Blackburn, 1978) requiring longer hydrolysis intervals. Those amino acids with polar side chains such as serine and threonine appear to be progressively destroyed during acid hydrolysis (Rees, 1946). For absolute accuracy in determination of the amino acid composition of a protein, corrections for losses of amino acids during hydrolysis or for the further release of amino acids from resistant bonds is required. The simplest and most common method of correction is to carry out the hydrolysis over several different intervals of time and to extrapolate assuming first order kinetics (Hirs *et al.*, 1954). Based on this latter technique correction factors can be determined (Kohler and Palter, 1967; Tkachuk and Irvine, 1969; Davies and Thomas, 1973; Slump, 1980; Gehrke *et al.*, 1985; Rowan *et al.*, 1992) and applied to the data from any subsequent single hydrolysis interval. The process of hydrolysis and degradation of amino acids occurs simultaneously, however, and a given point on a curve derived from the measurement of amino acids at different hydrolysis intervals, is not a true measure of amino acid yield. Where there are significant amino acid losses, extrapolations taken directly from points on an hydrolysis curve may be biased.

An alternative method for accurately determining the amino acid composition of a protein has been proposed (Robel and Crane, 1972), which uses a non-linear least squares regression of amino acid composition data from a series of hydrolysis intervals. Although this latter method appears to provide a more accurate estimation of the amino acid composition of a protein, the technique prescribes the analysis of five replicates at each of five different hydrolysis intervals ranging from 4 to 141 hours. This limits its routine application both in terms of the cost and the convenience of analysis. In addition, the technique has only been applied to a purified protein, and may require modification and further validation before it can be applied to samples such as human milk that contain not only a mixture of proteins, but also free amino acids.

**Analytical Methods**: The released amino acids are most often quantified after separation by ion-exchange chromatography (IEC) followed by detection with ninhydrin (Moore and Stein, 1951) on a dedicated amino acid analyser. Reverse-phase high-performance liquid chromatography (HPLC) columns have also been used, in conjunction with pre-column derivitisation methods, to separate and quantify most

of the amino acids in protein hydrolysates. Kan and Shipe (1981) compared HPLC with ion-exchange separation and reported that for a variety of food protein hydrolysates, similar amino acid values were obtained with both methods. Analysis of amino acids in a sample using Gas-Liquid Chromatography (GLC) has not been successful until recently when *t*-butyldimethyl-silylation yielded stable derivatives for all amino acids (Goh *et al.*, 1987). Although IEC is the currently accepted method for amino acid determination, it is likely that in the future HPLC or GLC, whereby amino acids can be determined with high sensitivity and fast throughput of samples, will become more prevalent.

### 1.4.6 Summary of compositional data on the amino acids in human milk

There have been many studies of the amino acid composition of human milk with the Committee on Nutrition (1985) reviewing over 200 independent studies to produce an average amino acid composition of human milk. Taking into consideration all of the factors that can influence the amino acid composition of human milk and its accurate determination, however, the number of estimates that can be considered representative and accurate is significantly reduced. A comprehensive study involving the collection of human milk from 200 women (Department of Health and Social Security[6], 1977), is often referred to as providing the definitive estimate of the amino acid composition of human milk. This study was thorough and comprehensive in the collection, storage and analysis of the milk samples. Although the authors stated that the amino acid composition data were corrected for hydrolytic losses, no detail was given as to which amino acids were corrected and what method of correction was used. Lemons *et al.* (1983), in determining the amino acid composition of human milk, attempted to correct for losses for all of the amino acids in human milk during acid hydrolysis. In their study a known mixture of amino acids present in protein was prepared from individual pure amino acid standards, subjected to hydrolysis, and the percentage recovery measured. Each amino acid in a human milk sample, that had been hydrolysed for 24 hours, was corrected using the percentage recoveries. Although correction for losses of most amino acids in a protein is worthwhile (Robel and Crane, 1972), it is likely that the rate of destruction of free amino acids in a pure sample will differ from

[6] *Hereafter DHSS (1977)*

**Table 1.4.**

*The amino acid composition of mature human milk (mg/100g protein)**

| Amino Acid | Reference+ | | | | | | | Mean |
|---|---|---|---|---|---|---|---|---|
| | a | b | c | d | e | f | g | |
| Aspartic Acid | 102.5 | 90.1 | 87.7 | 91.4 | 94.6 | 80.8 | 94.5 | 91.6 |
| Threonine | 44.2 | 49.3 | 46.2 | 44.6 | 49.9 | 49.7 | 48.2 | 47.4 |
| Serine | 61.0 | 46.5 | 43.0 | 45.7 | 46.4 | 55.9 | 49.1 | 49.7 |
| Glutamic Acid | 203.2 | 184.8 | 179.3 | 194.3 | 168.5 | 163.8 | 194.6 | 184.1 |
| Proline | 70.7 | 84.4 | 95.6 | 94.9 | 76.5 | 91.6 | 78.8 | 84.6 |
| Glycine | - | 22.8 | 25.5 | 22.9 | 23.2 | 24.1 | 21.3 | 23.3 |
| Alanine | 30.9 | 37.0 | 41.4 | 37.7 | 44.7 | 41.2 | 34.3 | 38.2 |
| Valine | 61.8 | 69.2 | 69.3 | 56.0 | 77.4 | 55.1 | 60.2 | 64.2 |
| Isoleucine | 60.1 | 57.8 | 53.4 | 54.9 | 57.6 | 77.6 | 53.8 | 59.3 |
| Leucine | 88.3 | 91.9 | 95.6 | 100.6 | 94.6 | 112.6 | 97.3 | 97.3 |
| Tyrosine | 53.9 | 46.5 | 30.3 | 38.9 | 32.7 | 48.9 | 42.6 | 42.0 |
| Phenylalanine | 42.4 | 37.9 | 38.3 | 36.6 | 37.8 | 40.4 | 39.9 | 39.0 |
| Histidine | 19.3 | 22.8 | 24.7 | 22.9 | 26.7 | 25.6 | 21.3 | 23.3 |
| Lysine | 64.5 | 66.4 | 71.7 | 65.1 | 67.9 | 77.6 | 63.0 | 68.0 |
| Arginine | 39.8 | 40.8 | 39.0 | 34.3 | 42.1 | 40.4 | 41.7 | 39.7 |
| Cysteine | 19.4 | 22.8 | 19.9 | 26.3 | 21.5 | - | 21.3 | 21.9 |
| Methionine | 22.1 | 11.4 | 15.1 | 12.6 | 16.3 | - | 18.5 | 16.0 |
| Tryptophan | 15.9 | 18.0 | 23.9 | 20.9 | 21.5 | 14.8 | 19.5 | 19.2 |

* Including free amino acids and peptides.

\+ References: a. Macy and Kelly (1961); b. Geigy, 1977; c. DHSS (1977); d. Svanberg *et al.* (1977); e. Casey and Hambidge (1981), f. Lemons *et al.* (1983), g. Committee on Nutrition (1985).

that of amino acids in a protein mixture especially if it contains nonproteinaceous components (Blackburn, 1978). The method described by Lemons *et al.* (1983) has also been used to correct for losses of amino acids during the acid hydrolysis of sow's milk (Wu and Knabe, 1994). Despite near identical hydrolytic conditions, different recoveries for each of the amino acids in the standard mixture were reported by Lemons *et al.* (1983) and Wu and Knabe (1994), suggesting that further validation of this method is required before it can be routinely applied.

A range of current estimates of the amino acid composition of human milk is given in Table 1.4. The variability of these estimates suggests that a comprehensive study of the composition of human milk is required using strict criteria for collection, handling and chemical analysis of the milk samples including the correction for losses of most of the amino acids in human milk.

## 1.5 NON-NUTRITIONAL ROLE OF HUMAN MILK PROTEINS

In addition to providing nutrients to the infant, human milk, like the milk from other mammals is known to contribute to protection of the recipient infant from a number of gastrointestinal and respiratory infections (Hanson and Winberg, 1972; Welsh and May, 1979; Casey, 1989). Many antimicrobial substances have been identified in human colostrum and milk (Reddy *et al.*, 1977; McClelland *et al.*, 1978; Suzuki *et al.*, 1983; Bratanov and Hristova-Koleva, 1984) and the different functions of human milk proteins, and in particular the immune proteins, within the gastrointestinal tract of the human infant have been reviewed (Welsh and May, 1979; Blanc, 1981; Lönnerdal, 1985; Casey, 1989; Koldovsky, 1989a). This has direct implications for the determination of amino acid requirements for infants, in that many of the proteins in human milk thought to have a protective role may resist digestion. Therefore, the gross amino acid composition may not reflect the amino acids in human milk absorbed by the breast-fed infant. Estimates of the amino acid requirements of infants may need to be reassessed to take into consideration the digestibility of the milk proteins in human milk. The role of different milk proteins in the infant, their relative resistance to digestion and prediction of the digestible amino acid composition of human milk will now be discussed.

**Table 1.5.**

*The protein content of mature* human milk (g/100 ml)*

| | Reference+ | | | | | |
|---|---|---|---|---|---|---|
| | a | b | c | d | e | Mean |
| *Total Protein* | 0.88 | 0.89 | - | 0.88 | 0.90 | 0.89 |
| **Casein** | 0.31 | 0.25 | 0.20 | 0.25 | 0.25 | 0.25 |
| **Whey** | 0.57 | 0.64 | 0.60 | - | 0.65 | 0.62 |
| α-lactalbumin | 0.15 | 0.26 | 0.30 | 0.25 | 0.26 | 0.24 |
| Serum albumin | 0.04 | 0.05 | 0.03 | 0.03 | 0.05 | 0.04 |
| Lactoferrin | 0.15 | 0.17 | 0.17 | 0.15 | 0.18 | 0.16 |
| Lysozyme | 0.05 | 0.05 | 0.04 | 0.02 | 0.05 | 0.04 |
| Secretory IgA# | 0.10 | 0.10 | 0.10 | 0.08 | 0.10 | 0.10 |
| IgG§ | - | 0.003 | 0.003 | 0.001 | 0.003 | 0.003 |
| IgM¶ | - | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 |
| Others | 0.007 | - | - | - | - | 0.007 |

* 2nd-6th month of lactation.
\+ References: a. Gurr (1981); b. Blanc (1981); c. Friend *et al.* (1983); d. Lönnerdal (1985); e. van Woelderen (1987).
\# Immunoglobulin A.
§ Immunoglobulin G.
¶ Immunoglobulin M.

### 1.5.1 Proteins in human milk and their possible function

Estimates of the concentration of different proteins in mature human milk, reported in several reviews, together with the mean values are given in Table 1.5. As it is difficult to separate and quantify human casein, the exact casein content of human milk is not known (Kunz and Lönnerdal, 1992). The most common method for the precipitation of casein, adopted from the analysis of bovine milk casein, leads to varying amounts of the whey proteins, such as lactoferrin (LF), serum albumin or secretory immunoglobulin A (sIgA) becoming entrapped in the casein pellet (Nagasawa *et al.*, 1974; Kunz and Lönnerdal, 1990). Several studies have determined the ratio of casein protein:whey protein in human milk, with a range of values from 10:90 (Kunz and Lönnerdal, 1992) and 20:80 (Harzer *et al.*, 1986) to 30:70 (Lönnerdal and Forsum, 1985), 40:60 (Kunz and Lönnerdal, 1990, 1992) and 45:55 (Harzer *et al.*, 1986) being reported for mature milk. Based on the values reported in Table 1.5, the casein:whey

ratio of human milk appears to be approximately 30:70, although it should be noted that further investigation of the casein content of human milk is required.

Casein, α-lactalbumin and serum albumin in human milk are primarily providers of amino acids to the infant. Casein is known to be a carrier of calcium (Jenness and Sloan, 1970; Kitts and Yuan, 1992) and may help in the absorption of calcium in the neonate (Lee *et al.*, 1983; Sato *et al.*, 1986). Kappa-casein has also been shown to stimulate the growth of *Bifidobacterium infantis* (Azuma *et al.*, 1984). The growth was more pronounced after treatment of the k-casein with either pepsin or chymosin, however, suggesting that the stimulatory effect was a by-product of casein digestion rather than a primary function *per se*. The protein α-lactalbumin is part of the enzyme, lactose synthase, which is responsible for lactose synthesis in the mammary gland (Blanc, 1981) and has an excellent nutritive value (Forsum, 1973), with a high content of lysine and cysteine and a particularly high tryptophan content (Heine et al, 1991). Alpha-lactalbumin has been found to bind calcium in a 1:1 molar ratio (Lönnerdal and Glazier, 1985), and may therefore facilitate the absorption of calcium in the infant. Human milk serum albumin, being identical to the serum albumin in blood plasma, does not appear to be synthesised in the mammary gland. It is thought that the very small proportions of serum albumin transferred into the milk from the blood, provide a further source of amino acids to the infant.

The proteins, sIgA, LF and lysozyme, identified as having some degree of immunological function within the digestive tract of the infant, account for over 30% of the total protein in mature human milk. Secretory IgA appears to act locally in the infant's gastrointestinal tract by binding to pathogens, thereby preventing these microorganisms from attaching to the mucosal lining and colonising the infant's gut (Lönnerdal, 1985).

Lactoferrin is thought to act bacteriostatically in the infant's gastrointestinal tract by binding iron, a nutrient also required by pathogenic organisms (Blanc, 1981; Lönnerdal, 1985). In conjunction with this action, LF may facilitate the absorption of iron by the infant. This is supported by observations that the bioavailability of iron in human milk is considerably higher than the availability of iron in bovine milk (Saarinen *et al.*, 1977). A LF receptor has been found in the small intestine of the Rhesus monkey (Davidson and Lönnerdal, 1985a) and human intestine (Cox *et al.*, 1979), that may enhance the uptake of iron from LF into the mucosal cells. It has been suggested that receptor-mediated uptake of LF-bound iron is responsible for the high bioavailability

of iron in human milk.

Human milk contains a higher concentration of lysozyme compared to the milks of most other mammalian species (Chandan *et al.*, 1968). This enzyme catalyses the hydrolysis of ß-1,4-glycoside bonds, which are part of the cell wall of gram-positive bacteria (Blanc, 1981). Therefore, it has been suggested that lysozyme in human milk has a bacteriostatic function in the gastrointestinal tract of breast-fed infants similar to lysozyme's role in tear secretions in the eye (Lönnerdal, 1985).

There are a number of other components in human milk such as bile-salt stimulated lipase (Blanc, 1981), α-amylase (Lönnerdal, 1985), epidermal growth factor (Koldovsky, 1989a), nucleotides (Janas and Picciano, 1982) and various hormones (Koldovsky *et al.*, 1989) that are also thought to have some functional role within the infant's gastrointestinal tract. In addition, milk proteins are a rich source of biologically active peptides such as casomorphins and caseinophosphopeptides, formed *in vivo* during protein digestion (Zioudrou *et al.*, 1979; Loukas *et al.*, 1983; Brantl, 1984; Meisel and Frister, 1989). Although the role of casomorphins is not yet fully understood, evidence exists that casomorphins may act as exogenous regulators of gastrointestinal motility and hormone release (Meisel and Frister, 1989).

### 1.5.2 Digestion of the human milk proteins

#### *1.5.2.1 The digestion and absorption of protein*

The digestion and absorption of protein, comprising the combined action of several proteolytic enzymes and different mechanisms for absorption, has been the subject of detailed review (Gitler, 1964; Cuthberston and Tilstone, 1972; Erbersdobler, 1973; Snook, 1973; Rérat *et al.*, 1976; Adibi and Kim, 1981; Matthews, 1983; Britton and Koldovsky, 1989). In the human adult, protein digestion occurs predominantly in the stomach and proximal small intestine and the main site of absorption is the distal small intestine. Undigested proteins leaving the small intestine are metabolized by the microbial population in the large intestine.

**Protein digestion and absorption in the stomach**: Protein digestion begins in the stomach by the gastric proteinase, pepsin, in the presence of hydrochloric acid, which produces the low pH necessary for peptic activity. The main products of gastric digestion are large polypeptides and undigested or only very partially digested protein, with a small proportion of oligopeptides and free amino acids (Matthews, 1983). Only the oligopeptides and free amino acids are ready for absorption (in the small intestine).

Consequently, the major part of protein digestion must occur in the intestine.

**Protein digestion and absorption in the small intestine**: After entering the intestinal lumen, the partially digested protein is mixed with pancreatic, intestinal and bile secretions, all of which are alkaline and thus terminate the activity of pepsin and provide the optimal pH range required by the intestinal proteinases. Proteinaceous material is digested by pancreatic enzymes, by enzymes in the brush border of intact epithelial cells, by brush border enzymes shed into the lumen, and by peptidases of desquamated cells.

The pancreatic enzymes can be classified into two groups (Kidder and Manners, 1978): Endopeptidases (trypsin, chymotrypsin and elastase) act on susceptible interior peptide bonds wherever they occur within the protein. The carboxypeptidases, which cleave an amino acid residue from the carboxyl end of the protein chain, are classified as exopeptidases. Perhaps the most crucial enzyme for the process of intraluminal proteolysis, however, is an enteropeptidase (located on the brush border of the small intestinal mucosa), which is responsible for the initial activation of trypsinogen (the inactive zymogen of trypsin) to trypsin. In turn, this leads to the activation of other pancreatic enzymes. Following pancreatic enzyme digestion, oligopeptides are digested by aminopeptidases, also classified as exopeptidases, present at both the brush border of the intestinal mucosa and within the mucosal cell. These latter peptidases act by removing an amino acid residue from the amino-end of the oligopeptides. Intestinal enzyme activities have been observed to be regionally distinct. For example, alkaline phosphatase is predominant in the duodenum, disaccharidases are predominant in the jejunum, and aminopeptidases are predominant in the ileum (Lindberg *et al.*, 1975), suggesting that the completion of carbohydrate digestion occurs in the middle of the small intestine, and the digestion of proteins is nearly complete at the end of the small intestine.

Both amino acids and peptides are absorbed from the intestinal lumen by independent mechanisms (Adibi and Kim, 1981), though amino acids liberated in the brush border by partial hydrolysis of peptides will compete for uptake with free amino acids (Matthews, 1983). Intestinal perfusion experiments suggest that both amino acids and peptides are most rapidly absorbed in the jejunum (Adibi, 1985).

**Protein digestion and absorption in the large intestine**: In the large intestine, there is considerable bacterial deamination, decarboxylation and transformation as well as synthesis of bacterial protein from amino acids arriving from the small intestine

(Fauconneau and Michel, 1970; Wrong *et al.*, 1981; McNeil, 1988). It is assumed that amino acids are not absorbed in nutritionally significant amounts from the large intestine, with the products of bacterial degradation of protein being absorbed in the form of ammonia together with a small amount of amines and amides, which are ultimately excreted mainly as urinary urea (McNeil, 1988).

### *1.5.2.2 Digestion of milk proteins in infancy*

There have been several reviews of the digestion of protein in infancy (Koldovsky, 1978; LIndberg *et al.*, 1989). Lindberg *et al.* (1989) described how gastric juice collected from term infants hydrolysed various milk proteins efficiently at pH 1.5. There was no peptic hydrolysis of proteins at pH 4.5, however, which is the pH expected to occur in the stomach of an infant during a milk-feeding. No substantial digestion of protein was found in specimens of gastric contents taken from 5- to 8-day old infants (Mason, 1962), which supports this finding. In older infants (2-6 weeks of age), however, traces of hydrolyzed protein were found in stomach contents, with cow's milk protein being hydrolysed to a greater extent than the proteins in human milk (Koldovsky, 1978). Controversy exists concerning the occurrence of rennin (a peptidyl peptide hydrolase) in the infant's stomach (Koldovsky, 1978). This enzyme coagulates milk at pH values between 6.0 and 6.5. Thus if the pH of the gastric contents is increased by a large amount of milk, rennin may become effective. Although this enzyme has not been identified in the human infant's stomach, the process leading to precipitation of protein in the infant's stomach is very effective with breast-milk casein precipitating within 30 minutes of feeding (Mason, 1962).

By three months of age the infant's pancreatic and mucosal proteolytic enzyme activity is approaching adult levels (Koldovsky, 1978; Lindberg *et al.*, 1989). There have been very few studies on the intestinal digestion of protein in infants (Feinstein and Smith, 1951, cited by Lindberg *et al.*, 1989; Borgström *et al.*, 1960; Hirata *et al.*, 1965; Llanes et al, 1987). Most of the recent studies have been *in vitro* investigations of the proteolytic capacity of gastrointestinal fluid collected from infants (Lindberg, 1974; Jakobsson *et al.*, 1982) and weanling rats (Britton and Koldovsky, 1987). Jakobsson *et al.* (1982) examined the initial stages of hydrolysis of various human and bovine milk proteins by the duodenal juice from term infants. The relative digestion of the bovine milk proteins was: casein > ß-lactoglobulin > α-lactalbumin and for the human milk proteins, α-lactalbumin > lactoferrin. The digestibility of human milk proteins in

luminal fluid collected from weanling rats (Britton and Koldvosky, 1987) produced a similar ranking of: human ß-casein > α-lactalbumin > lysozyme > lactoferrin. Llanes *et al.* (1987) collected samples of jejunal contents via a jejunostomy in infants fed human milk, and showed that both human and bovine caseins were efficiently hydrolysed by the human infant.

Secretory IgA is quite resistant to proteolysis, more so than its serum counterpart, apparently due to sIgA's unique structure with the secretory component and J chain joining the two IgA molecules (Lindh, 1975). Parkin *et al.* (1973) demonstrated that the action of trypsin produced no detectable changes in the agglutination of a wide range of aerobic intestinal bacteria by sIgA.

Lactoferrin can exist in either an iron-saturated form or free of iron (apo-lactoferrin), with the iron-saturated form being more resistant to digestion (Brock *et al.*, 1976). Although LF in human milk is only saturated to a very limited extent, 3-5% of total iron-binding capacity (Fransson and Lönnerdal, 1980), it has been suggested that iron present in other compartments may be released and transferred to LF (Lönnerdal, 1985), thus increasing LF's resistance to digestion. Lönnerdal (1985) also suggested that iron-saturated LF may attach to a specific brush-border receptor, releasing iron for absorption. The apo-lactoferrin would subsequently be digested.

Protease inhibitors have been detected in the milk of several mammalian species. As part of the transfer of immunity, piglets absorb undegraded or partially degraded colostrum proteins via the intestinal epithelium into the blood during the first 24-36 hours after birth (Pond and Houpt, 1978). A trypsin inhibitor present in high concentrations in sow's colostrum, and thought to limit proteolysis of the immune proteins (Carlsson *et al.*, 1980), disappears from the milk by 5-7 days of lactation (Weström *et al.*, 1982). Protease inhibitory factors have also been found in human colostrum (Lindberg, 1979), although they appear to be similar to those in plasma and other body fluids, rather than specific components of mammary secretions as in the sow (Britton and Koldovsky, 1989). The efficacy of human milk protease inhibitors in reducing proteolysis in the infant's gastrointestinal tract has not been fully investigated, although an *in vitro* study demonstrated that the protease inhibitors were resistant to proteolysis by pepsin and pancreatic enzymes (Davidson and Lönnerdal, 1990).

There is sufficient evidence, therefore, to suggest that the immune proteins are at least partly resistant to digestion in the infant's gastrointestinal tract. This is supported by the detection of intact immune proteins in the faeces of exclusively

breast-fed infants (Ogra *et al.*, 1977; Spik *et al.*, 1982; Jatsyk *et al.*, 1985; Davidson and Lönnerdal, 1985b, 1987; Schanler *et al.*, 1986; Prentice *et al.*, 1987; Donovan *et al.*, 1989; Prentice *et al.*, 1989; Goldman *et al.*, 1990). A summary of these studies is given in Table 1.6.

**Table 1.6.**

*Faecal excretion (expressed as a percentage of intake) of protective proteins in term and preterm infants*

| Protein | Infants | | % of Intake | Reference |
|---|---|---|---|---|
| | Age | Number | | |
| **Lactoferrin** | 6 wks* | 33 | 3 | Schanler *et al.*, 1986 |
| | 6 wks | 10 | 1 | Prentice *et al.*, 1987 |
| | 12 wks | 10 | 0.6 | Prentice *et al.*, 1987 |
| | 12 wks | 2 | 0.4-1.6 | Davidson & Lönnerdal, 1987 |
| | 12 wks | 11 | 1.5 | Prentice *et al.*, 1989 |
| | preterm | 14 | 3 | Donovan *et al.*, 1989 |
| **sIgA** | 18-72 hrs | 7 | 79 | Ogra *et al.*, 1977 |
| | 6 wks* | 33 | 9 | Schanler *et al.*, 1986 |
| | 6 wks | 10 | 17 | Prentice *et al.*, 1987 |
| | 12 wks | 10 | 11 | Prentice *et al.*, 1987 |
| | 12 wks | 2 | 10-35 | Davidson & Lönnerdal, 1987 |
| | 12 wks | 11 | 6-11 | Prentice *et al.*, 1989 |
| | preterm | 14 | 9.5 | Donovan *et al.*, 1989 |
| **Lysozyme** | 6 wks* | 33 | 0.1 | Schanler *et al.*, 1986 |
| | preterm | 14 | 3.5 | Donovan *et al.*, 1989 |

* Very low birth weight infants.

It has also been demonstrated, however, that both breast-fed and formula-fed infants excrete endogenous sIgA and LF into their gastrointestinal tract (Haneberg and Tonder, 1973; Koutras and Vigorita, 1989). The occurrence of endogenous immune proteins, undoubtly accounts for some of the faecal sIgA and LF found in the studies discussed above, but does not explain the significantly higher levels of sIgA and LF found in the faeces of breast-fed infants as compared to formula-fed infants (Spik *et al.*, 1982). Although it would appear that human milk immune proteins are resistant to digestion, another possibility is that the feeding of human milk stimulates a higher excretion of endogenous immune proteins by the infant (Schanler *et al.*, 1986; Koutras and Vigorita, 1989; Goldman *et al.*, 1990). Goldman *et al.* (1990) found that in very low birth weight infants, urinary LF excretion increased in infants fed human milk. Hutchens *et al.* (1991), however, found evidence to suggest that in very low birth weight infants undegraded LF of maternal origin was absorbed by the infant's gut and excreted intact in the urine. It remains unclear, therefore, whether the higher concentrations of immune proteins in the faeces of breast-fed infants are of maternal origin and have survived passage through the infant's gastrointestinal tract or whether their production by the infant has been enhanced as a result of stimulating factors present in breast milk.

Assuming that some of the immune proteins of maternal origin are resistant to digestion, their presence in the faeces of breast-fed infants will not give a true indication of their resistance to digestion. This is due to the considerable microbial breakdown of proteinaceous material in the large intestine (McNeil, 1988), thus the amount of immune protein entering the large intestine of breast-fed infants may be greater than that detected in the faeces. As much as 32% of ingested LF has been detected in the stools of ileostomised infants fed human milk (Hambraeus *et al.*, 1988). When this value is compared with the values of 1-3% presented in Table 1.6, the degree of bacterial breakdown occurring in the large intestine becomes evident.

### 1.5.3 The profile of amino acids in human milk which are available for nutrition

In summary, therefore, it would appear that some of the proteins in human milk are not digested. This has direct implications for the determination of amino acid requirements for infants, should the amino acid composition of human milk be used as a standard. The absorbable rather than gross amino acid composition of human milk may be a better basis from which to determine the amino acid requirements of

human infants.

Hambraeus *et al.* (1984) and Räihä (1985) concluded that the amount of digestible protein in human milk could be as low as 0.7g/100 ml, assuming that none of the immune proteins were digested. Harzer *et al.* (1987) calculated the amino acid composition of the protein fraction thought to be totally digested. The amount and amino acid composition of the digestible protein appeared to remain constant throughout the lactational period, and it was suggested that this fraction represents the true amino acid requirements of the infant, particularly since the amino acid composition of this fraction appears to be constant regardless of the stage of lactation. When the gross and digestible amino acid compositions of human milk were compared, Harzer *et al.* (1987) noted that for some amino acids the digestible fraction was only half of the total, whereas with other amino acids no or only minor changes occurred. Harzer *et al.* (1987) concluded that a re-evaluation of the current recommendations on protein requirements during infancy was necessary, specifically with respect to the individual amino acid requirements.

Using the amino acid compositions of the major proteins in human milk (total casein, α-lactalbumin, lactoferrin, lysozyme, IgA and serum albumin), together with the known proportions of these proteins in human milk (Table 1.5), and the anticipated digestibility of each protein, a profile of the gross and digestible amino acid compositon of human milk can be determined (Table 1.7). There are significant changes in the profile of amino acids depending on what degree of digestibility is assumed for the immune proteins. Based on the calculations used to derive the data in Table 1.7, the digestible protein content of human milk may range from 0.59-0.86g/100ml assuming a gross protein content of 0.89g/100ml. Thus a 6 kg 3-month-old human infant consuming 900 ml of breast milk per day would have a protein intake of between 0.89-1.3 g/kg body weight/day which is considerably lower than the several estimates of protein requirements given in Table 1.2, and on the lower end of the estimates of protein requirements based on breast-milk intake, also given in Table 1.2.

**Table 1.7**

*The gross and digestible amino acid composition of human milk*

*(g amino acid/100 g total protein)*

| Amino Acid | Gross* | Digestible+ | Digestible# |
|---|---|---|---|
| Aspartic Acid | 10.2 | 6.9 | 9.9 |
| Threonine | 4.8 | 2.8 | 4.7 |
| Serine | 5.5 | 3.1 | 5.3 |
| Glutamic | 15.2 | 11.3 | 14.8 |
| Proline | 6.8 | 5.0 | 6.6 |
| Glycine | 3.0 | 1.5 | 2.9 |
| Alanine | 4.3 | 2.4 | 4.1 |
| Valine | 5.1 | 3.0 | 4.9 |
| Isoleucine | 5.4 | 4.6 | 5.3 |
| Leucine | 10.0 | 7.0 | 9.7 |
| Tyrosine | 4.7 | 3.2 | 4.7 |
| Phenylalanine | 4.3 | 2.4 | 4.1 |
| Histidine | 1.8 | 1.3 | 1.8 |
| Lysine | 7.5 | 5.4 | 7.3 |
| Arginine | 4.6 | 2.2 | 4.3 |
| Cysteine | 3.5 | 2.4 | 3.4 |
| Methionine | 1.6 | 1.3 | 1.6 |
| Tryptophan | 1.9 | 1.0 | 1.8 |
| Total | 100.0 | 66.6 | 97.0 |

* Calculated using the individual amino acid compositions of total casein, α-lactalbumin, lactoferrin, lysozyme and serum albumin (Lönnerdal, 1989), IgA (Räihä, 1989), and the proportions of each protein as described in Table 1.5.

\+ Assuming that secretory IgA and lactoferrin are completely undigested.

\# Assuming that 14% and 1.5% of secretory IgA and lactoferrin, respectively, escape digestion in the gastrointestinal tract of 12 wk old breast-fed infants (see Table 1.6).

## 1.6 DETERMINING THE PROFILE OF DIGESTIBLE AMINO ACIDS IN HUMAN MILK

There is sufficient evidence, therefore, to suggest that a reassessment of the amino acid requirements of human infants is required. The digestible rather than gross amino acid composition of human milk would appear to provide a better estimate of the infant's amino acid requirements. There are two possible approaches to determining the digestible amino acid composition of human milk. The first is to investigate the plasma amino acid concentrations in breast-fed infants. Although this method is relatively easy to carry out, it has several inherent limitations. Alternatively, the digestibility of the amino acids in human milk can be determined. This latter method is more difficult to conduct with infants, often dictating the use of model animals, which by their very nature, have limitations.

### 1.6.1 Plasma amino acid profiles

Investigation of plasma amino acid concentrations has primarily been used to compare the amino acid metabolism of breast-fed and formula-fed infants (Lindblad *et al.*, 1978; Lucas *et al.*, 1981; Tikanoja, 1982; Volz *et al.*, 1983; Ginsburg *et al.*, 1984; Janas *et al.*, 1987; Schanler and Garza, 1987; Rigo *et al.*, 1989; Lönnerdal and Chen, 1990) due to growing concern that the protein level commonly used for infant formulas, 1.5-1.6 g/100 ml, is higher than necessary and may actually impose some risks to the infant (Järvenpää *et al.*, 1982a, 1982b; Räiha *et al.*, 1986a, 1986b). In one study (Picone *et al.*, 1989), three formulas were prepared using bovine proteins, to have amino acid compositions very close to that of human milk. Picone *et al.* (1989) found, however, that providing formulas with an amino acid pattern and content similar to that of human milk did not produce a plasma amino acid pattern identical to that of the breast-fed infant. There are a number of possibilities as to why this occurred, one of them being the lower amounts of digestible amino acids in human milk compared to the formula.

The concentrations of amino acids in the plasma of breast-fed infants, could, in theory be used to determine the profile of digestible amino acids in human milk by developing a formula that produced a profile of amino acids in plasma the same as that observed in breast-fed infants. From this it could be assumed that the amino acid composition of the formula reflected the digestible amino acid composition of human milk.

The measurement of plasma amino acid concentrations, however, does not give a direct quantitative estimate of amino acid absorption. Post-prandial measurements are limited by the time of sampling and other complex interrelationships between the intake of amino acids and the plasma amino acid concentration. For example, breast-fed and formula-fed infants have different postprandial responses, with plasma amino acid concentrations peaking 30 minutes and 60 minutes after a meal in breast-fed and formula-fed infants, respectively (Tikanoja, 1982). These differences are probably due to the lower protein intake and more rapid gastric-emptying time (Cavell, 1981) in the breast-fed infant. Most studies, therefore, measure the preprandial amino acid concentration in plasma. This latter method provides an indication of the infant's protein status, however, rather than a direct measure of absorbed amino acids. Two further aspects that need to be considered with this method are that metabolic synthesis and degradation of amino acids occurs within the gut mucosa and in the liver prior to blood sampling (LeLeiko, 1984), and the amino acid pattern that is measured in the plasma is the result not only of dietary protein but also of the endogenous gastrointestinal protein that is buffering or diluting the dietary protein (LeLeiko, 1984). In summary, although plasma amino acid concentrations may allow relative comparison between infants, they are not suitable for determining the digestible amino acid composition of human milk.

### 1.6.2 Digestibility of human milk proteins

An alternative approach is to determine the amino acid digestibility of human milk. Digestibility is defined as the difference between the amount of an amino acid ingested in the diet and that in the digesta collected at the end of the small intestine (ileal) or in the faeces, expressed as a proportion of the amount ingested.

#### *1.6.2.1 Faecal and ileal digestibility assays*

The traditional and simplest method for determining amino acid digestibility is based on measurement of faecal amino acid excretion (Sarwar, 1987). As described in section 1.5.2.1, amino acids and peptides released by digestion are absorbed in the small intestine, with oligopeptides being further hydrolysed intracellularly (Davenport, 1982). Macromolecular absorption of proteins, reported in preterm infants (Axelsson *et al.*, 1989; Hutchens *et al.*, 1991) appears to largely cease in early postnatal life (Karlsson *et al.*, 1985). Protein, peptides and amino acids, of either dietary or

endogenous origin, remaining unabsorbed at the end of the small intestine (terminal ileum), pass into the large intestine where they are subject to the action of a significant population of microbes (McNeil, 1988). It is as a result of this microbial metabolism, that estimates of amino acid digestibility based on faecal measurement are ambiguous. Studies in pigs have shown that there is generally a net breakdown of the amino acids entering the large intestine (Just, 1980), although for some amino acids, notably methionine and lysine, net synthesis may occur (Just, 1980; Low, 1980; Sauer and Ozimek, 1986). Therefore, determining digestibility based on the collection of faeces tends to overestimate the actual uptake of dietary amino acids. Measurement of amino acid flow and digestibility at the end of the ileum (Payne *et al.*, 1968) is now recognised as a more acceptable approach and is routinely used to determine the digestibility of dietary amino acids in animals (Low, 1980; Moughan and Smith, 1982; Sauer and Ozimek, 1986).

The ileal method, while apparently superior to the faecal digestibility technique, relies on the assumption that amino acids are not absorbed in nutritionally significant amounts from the large intestine. Nitrogen absorbed from the large intestine is thought to be in the form of ammonia together with small amounts of amines and amides, which are ultimately excreted mainly as urinary urea (Zebrowska, 1982; Just, 1983; McNeil, 1988). Whereas several studies have shown that amino acids are not absorbed in significant amounts in growing and adult animals (Zebrowska, 1973; Just *et al.*, 1981; Schmitz *et al.*, 1991), with the possible exception of the horse (Slade, 1971), there is some evidence for amino acid absorption from the large intestine of the neonate. James and Smith (1976) found that at birth the piglet proximal colon has a structure similar to that of the small intestine and speculated that absorption of amino acids in the large intestine of neonates may be of physiological importance. Furthermore, several *in vitro* studies demonstrated that methionine is absorbed into the proximal colonic epithelium of new-born piglets (James and Smith, 1976; Smith and James, 1976; Jarvis *et al.*, 1977). While there are no published quantitative data on the *in vivo* absorption of amino acids in human neonates, Heine *et al.* (1987) demonstrated absorption and retention of protein $^{15}N$ in the colon of human infants. In this latter study, however, it is likely that the protein $^{15}N$ was degraded by intestinal bacteria and absorbed as labelled ammonia. Assuming that amino acids are not absorbed in nutritionally significant amounts from the infant's large intestine, it would seem more appropriate to determine the ileal rather than faecal amino acid digestibility of human milk.

*1.6.2.2 Correction for endogenous amino acid excretion*

In determining the ileal digestibility of amino acids based on measurement of the flow of amino acids at the terminal ileum, differentiation needs to be made between undigested and unabsorbed dietary and endogenous proteins and amino acids. Digestibility values determined without taking into consideration the endogenous flows of amino acids are termed apparent, while digestibility values determined using amino acid flows that have been corrected for endogenous amino acid excretions are termed true.

Endogenous proteinaceous material consists of protein from digestive enzymes and mucus (Kidder and Manners, 1978). In addition, sloughed-off mucosal cells, endogenous urea and ammonia, and free amino acids appear in the gastrointestinal lumen (Rérat, 1981). Low (1985) estimated that endogenous protein may contribute up to 50% of the total amount of protein that enters the digestive tract of the pig. Only part of this endogenous protein would be recovered at the terminal ileum, however, as endogenous protein, like dietary protein would be partially digested and absorbed.

Traditionally, the endogenous flows of amino acids at the terminal ileum of an animal have been determined after feeding the animal a protein-free diet (Zebrowska and Buraczewska, 1972; Sauer *et al.*, 1977; Wilson and Leibholz, 1981c; de Lange *et al.*, 1989a; Wang and Fuller, 1989; Moughan and Schuttert, 1991; Butts *et al.*, 1991). The exclusion of protein from the diet may create a physiologically abnormal metabolism in the animal (Low, 1980), resulting in a reduced flow of endogenous amino acids. When pigs were fed a protein-free diet while receiving a well-balanced mixture of amino acids intravenously (de Lange *et al.*, 1989b), however, of all the amino acids measured, only the recovery of proline was significantly reduced. Proline is abundant in the protein collected from the terminal ileum of pigs fed protein-free diets (de Lange *et al.*, 1989a; Moughan and Schuttert, 1991; Moughan *et al.*, 1992b) and this elevated level of proline is thought to be directly related to the protein status of the animal.

Another approach to determining the endogenous flows of amino acids at the terminal ileum involved feeding rats a diet containing synthetic amino acids as the sole N source but devoid of the amino acid under consideration. The flows of amino acids determined using this method were not significantly different from those determined using the protein-free method (Skilton *et al.*, 1988; Darragh *et al.*, 1990). The endogenous protein and amino acid flows at the terminal ileum of an animal can also be determined by regression to zero protein intake using a series of levels of a test

source of protein (Carlson and Bayley, 1970). Leibholz and Mollah (1988) found that the regression technique generated similar values to those obtained after feeding pigs a protein-free diet.

The protein-free, synthetic amino acid and regression techniques rely on the assumption that the absence of dietary protein does not affect the recovery of endogenous protein and amino acids. There is evidence to suggest, however, that the presence of dietary peptides in the intestinal lumen is important in maintaining physiologically normal levels of digestive enzyme secretion (Snook, 1965; Fauconneau and Michel, 1970; Schneeman, 1982). Furthermore, it has been clearly demonstrated, using a variety of different experimental techniques (de Lange *et al.*, 1990; Darragh *et al.*, 1990; Moughan and Rutherfurd, 1990; Butts *et al.*, 1991) that feeding an animal a protein-free diet will lead to an underestimation of the endogenous flows of amino acids. Given that the regression technique generates similar values to those determined using the protein-free technique, the regression method may also underestimate the ileal endogenous amino acid excretion.

It would appear important, therefore, for endogenous amino acid excretions to be determined in animals that have been fed dietary protein. Accordingly, three further methods have been developed for the determination of endogenous amino acid flows under protein alimentation.

The isotope dilution method (Souffrant *et al.*, 1982), whereby an isotopically labelled amino acid tracer is infused into the animal, allows a distinction to be made between labelled endogenous protein and unlabelled dietary protein. Moughan *et al.* (1992a) found, however, that in its present form the isotope dilution technique is unsuitable as a method for measuring endogenous N excretion in animals, due to the difficulty in identifying a suitable precursor pool for the endogenous N-containing material.

Guanidination of the lysine in a protein to homoarginine (Hagemeister and Erbersdobler, 1985; Moughan and Rutherfurd, 1990), before feeding the reacted protein to an animal, only allows the direct measurement of endogenous losses of lysine under protein alimentation.

An alternative approach proposed by Moughan *et al.* (1990a) and subsequently developed and evaluated with rats (Butts *et al.*, 1991) and the growing pig (Butts *et al.*, 1993), involves feeding the animal a semi-synthetic diet containing enzymically hydrolysed casein (EHC) as the sole N source. Ileal digesta are collected and

centrifuged followed by ultrafiltration of the supernate (molecular exclusion limit: 10,000 Da) to remove all dietary peptides and amino acids remaining unabsorbed at the end of the small intestine. The precipitate plus the high-molecular-weight fraction resulting from centrifugation and ultrafiltration, provides a measure of endogenous amino acid flows. Although this method appears to provide a better estimate of the endogenous amino acid excretion in animals under conditions of protein alimentation, further validation work is required.

### 1.6.3 Need for a model animal

To determine the digestible amino acid composition of human milk, it would appear necessary to determine the ileal amino acid digestibility of human milk, taking into consideration the endogenous excretion of amino acids. In practice, however, determination of the faecal amino acid digestibility of human milk with corrections for faecal endogenous amino acid excretions is the only practical method for use with human infants. Ileal digesta samples have been collected from infants by passing a thin vinyl tube into the gastrointestinal tract via the nose, and allowing it to proceed to the ileo-caecal area (Hirata *et al.*, 1965). No further applications of this technique in infants have been reported, however, probably due to its invasive nature. Sampling digesta from infants that have had ileostomies (Hambraeus *et al.*, 1988) is possible, although infants with ileostomies usually have compromised digestive function, and samples collected from these infants may not be representative of infants in general.

Determination of the true ileal amino acid digestibility of human milk, therefore, dictates the need for an acceptable model animal for the breast-fed human infant.

## 1.7 MODEL ANIMALS IN THE STUDY OF ASPECTS OF PROTEIN DIGESTION IN INFANTS

### 1.7.1 Choice of Model Animal

When choosing an animal model, it is important to consider both the similarities and differences between the animal and human in terms of anatomy, physiology, susceptibility to and aetiology of nutritional diseases, similarity of immunological responses, qualitative requirements for essential nutrients and patterns of growth in relation to the topic being studied. There have been numerous reviews on the use of animal models in human medical and nutritional research (Mitruka *et al.*, 1976; Waddell and Desai, 1981; deLemos and Kuehl, 1987; Low, 1992; Raju, 1992).

Rats, guinea pigs, dogs, swine and non-human primates are commonly used to study aspects of human nutrition and metabolism. The rat is omnivorous, easily handled and cared for, is a prolific breeder, and can thrive in small areas. For a long period after weaning, the rat continues to gain weight and is, therefore, useful in nutrition studies which monitor weight over extended periods of time. One of the main limitations to the use of the rat in nutrition research involves the magnitude of phsyiological differences when compared to the human. The rat requires different proportions of the essential nutrients, it is not generally susceptible to the same diseases, and it exhibits radically different patterns of growth and development compared to the human, making extrapolation difficult (Waddell and Desai, 1981). Dogs were classically used in physiological research due to gastric similarities to humans, and continue to be used in the study of gastric disorders in humans (Waddell and Desai, 1981). Various species of non-human primates are frequently used in biomedical research because of their anatomical similarities to humans. Aspects of infant nutrition and metabolism studied using non-human primates include foetal malnutrition (Fisher *et al.*, 1983), kwashiorkor-like diseases in children (Coward and Whitehead, 1972), paediatric enteral and parenteral regimens (Dempsey *et al.*, 1985), the effect of breast-feeding or formula-feeding on hormonal responses in infancy (Lewis *et al.*, 1993), and the development of organ blood flow in the neonate (Paton and Fisher, 1984). Lönnerdal *et al.* (1984) conducted a longitudinal study of the milk composition of the Rhesus monkey (*Macaca mulatta*), to determine whether that species was suitable as an animal model for milk composition and metabolism studies in humans. Lönnerdal and workers extended their work with the Rhesus monkey to include an investigation of the digestion of human milk and infant formulas (Llanes *et al.*, 1987), the occurrence of lactoferrin receptors in the intestinal mucosa (Davidson and Lönnerdal, 1985a), and the absorption of iron from human milk or formulas (Davidson *et al.*, 1990). They concluded that the Rhesus monkey was an excellent model for the human infant, although no direct comparison of the Rhesus monkey and human infant has been reported. Ausman *et al.* (1986) used Cebus monkeys (*Cebus albifrons*) to determine the protein quality of different infant formulas based on a slope-ratio assay. They found, however, that large variations in the data obtained from the monkeys made it difficult to determine whether the monkey was a better model than the rat for determining protein quality. Although non-human primates would appear to be a logical choice as an animal model for humans, they are expensive to purchase, breed

and maintain, which limits their routine use in nutritional studies (Mitruka *et al.*, 1976).

The pig is also frequently used as a model for research in human nutrition and related clinical disorders (Pond and Houpt, 1978; Fleming and Wasilewski, 1984; Latymer *et al.*, 1985; James *et al.*, 1986; Tumbleson, 1986; Lenaars and Moughan, 1993; Rowan *et al.*, 1994) and the suitability of the pig as a model animal for studying aspects of human nutrition has been extensively reviewed (Pond and Houpt, 1978; Dodds, 1982; Miller and Ullrey, 1987; Moughan and Rowan, 1989; Moughan *et al.*, 1992c; Moughan *et al.*, 1994). In addition to being similar to humans in several aspects related to its nutrition (Miller and Ullrey, 1987), the pig offers several logistical advantages (Moughan and Rowan, 1989) over the non-human primate. These include being readily available, inexpensive and being able to adapt to confinement. Pigs have a relatively short reproductive cycle, and it is possible to obtain genetically-defined lines of pigs. Sows usually produce large litters, making it easier to divide pigs of identical genetic background among several experimental groups. Like humans, the pig is an intermittent feeder and will consume a variety of foods similar to those eaten by man.

The piglet is also thought to be an appropriate model for studying aspects of human infant nutrition (Glauser, 1966; Book and Bustad, 1974; Mitruka *et al.*, 1976; Miller and Ullrey, 1987; Moughan *et al.*, 1992c; Innis, 1993; Borum, 1993; Shulman, 1993; Moughan *et al.*, 1994). Piglets readily adapt to bottle-feeding and have been used as models for infant nutrition by several workers (Schneider and Sarett, 1966; Cooper, 1975; Blakeborough *et al.*, 1983; Newport and Henschel, 1984, 1985; Goldstein *et al.*, 1986; Yao *et al.*, 1986; Shulman *et al.*, 1988; Bustamante and Lindgren, 1989; Jones *et al.*, 1990; Moughan *et al.*, 1990b; Moughan *et al.*, 1991).

### 1.7.2 Comparison of the piglet and human infant

#### *1.7.2.1 Digestive physiology*

The pig is markedly less-developed at birth than the human infant, but post-natally grows and develops more rapidly than the human and can be regarded (Mount and Ingram, 1971) as a fore-shortened and accelerated model of post-natal human growth and development. A comprehensive review of the piglet as a model animal for studying aspects of digestion and absorption in the milk-fed human infant has recently been made (Moughan *et al.*, 1992c).

At birth, piglets and human infants have similar sized digestive organs. If organ size is expressed relative to body weight, however, the piglet has a considerably

greater digestive capacity. The point at which the piglet and human infant are of similar bodyweight (5-6 kgs) occurs at the comparable developmental stage of peak lactation in the dam (three weeks in the sow and three months in the human). At this stage, however, the gut capacity of the piglet is still approximately double that of the human infant. In nutritional studies using the piglet as a model animal, this difference in gut capacity needs to be taken into consideration. Some differences also exist in the gastrointestinal tract anatomy of the two species (Moughan *et al.*, 1992c). There are regional differences in the anatomy, histology and morphology of the gastric mucosa. The piglet has separate entry points into the small intestine for bile and pancreatic secretions compared with a common opening in humans. There are also differences in the anatomical shape of the large intestine. None of these differences, however, are considered to be of major importance to digestive function in the two species.

One feature of the piglet's gastrointestinal development that will impact upon the usefulness of the piglet as a model, however, is the permeability of the piglet's intestinal epithelium to whole proteins in the first few days of life (Pond and Houpt, 1978). It is believed that the ability of the human gastrointestinal tract to exclude potentially antigenic intact dietary proteins increases with gestational age, and that gut closure occurs normally before birth (Karlsson *et al.*, 1985). Premature infants, however, appear to be able to absorb dietary protein intact (Hutchens *et al.*, 1991). Furthermore, recent studies have shown that the human infant is capable of transmitting intact dietary proteins from the gut to the blood for periods of up to three months after birth (Axelsson *et al.*, 1989). When deciding at what age the piglet is an appropriate model for the human infant, this phenomenon of gut closure needs to be considered. Karlsson *et al.* (1985) suggested that the pre-closure neonatal piglet may be an appropriate model for the premature human infant.

Although at birth there are quite large differences between the species for the activities of trypsin, sucrase and maltase, reflecting the greater degree of development in the human, the major digestive enzymes have similar activities in three-week-old piglets and three-month-old human infants. The relative activities of trypsin, sucrase and maltase remain, however, somewhat lower in the piglet (Table 1.8). In studies with miniature pigs, Shulman (1988) concluded that the degree of small intestinal maturation and the similar intestinal enzyme distribution compared with that of the human infant suggest that the miniature piglet is an excellent model for studies of intestinal enzyme development and regulation.

**Table 1.8.**

*Approximate relative activities of the major digestive enzymes in suckled or milk-fed human infants and piglets at birth and at the time of peak lactation in the dam (Moughan et al., 1992c)*

| | Activity, % of value at digestive maturity | | | |
|---|---|---|---|---|
| | human infant | | piglet | |
| Enzyme | birth | 3 months | birth | 3 weeks |
| Trypsin | 25-100 | 100* | 10 | 10 |
| Chymotrypsin | 30-55 | 100* | 60 | 100 |
| Pancreatic Lipase | 7-14 | 50* | 8 | 48 |
| Pancreatic amylase | 0-10 | 0-5 | 0 | 3-30 |
| Lactase | 100-400 | 100 | 300-600 | 100 |
| Sucrase | 100-200 | 100 | 0 | 25 |
| Maltase | 100 | 100 | 0-5 | 20 |
| Small-intestinal dipeptidases | 100 | 100 | 200 | 100 |

* Overall result from several highly variable sets of data.

Due to a lack of comprehensive observations made under suitable experimental conditions, inter-species comparison of gut microbiology is difficult. After studying more than 100 references on aspects of gastrointestinal microflora in healthy human infants and piglets, Moughan *et al.* (1992c) concluded that in the piglet, lactobacilli dominate the flora in the upper gastrointestinal tract and are an important component of the flora in the large intestine. In the human infant, although little is known about the bacteriology of the upper tract, in the large intestine *bifidobacteria* are probably the dominant organism, particularly in the breast-fed infant. It did not appear that there were any differences between the microflora of the piglet and human infant that would be of major nutritional significance.

*1.7.2.2 Comparison of the digestibility of milk protein in the piglet and human infant*

In several balance studies conducted with infants (aged 0-5 months old) fed different types of infant formula, the average absorption of dietary N from the digestive tract was 88% with a range of 87-90% (Fomon, 1960; Slater, 1961; Widdowson, 1965; Southgate and Barrett, 1966; Cordano *et al.*, 1988). The apparent faecal digestibility of N determined in piglets (aged 1-6 weeks) fed either reconstituted cow's milk (Braude *et al.*, 1970), or formulated milk diets (Widdowson, 1965; Carlson and Bayley, 1970; Leibholz, 1981; Wilson and Leibholz, 1981a, 1981b; Moughan *et al.*, 1990b) ranged from 86-98%, with an average of 96%. Lower values have been reported for the apparent ileal digestibility of N in piglets (Moughan *et al.*, 1990b). Similar ileal digestibility values are not available in the human infant for comparison with the piglet. Although both species are highly efficient at digesting milk protein, it would appear that the piglet may be slightly more efficient than the human infant. Direct comparison of the digestion of protein in both species is required, however, before a definitive conclusion can be drawn.

### 1.7.3 Summary

Based on a comparison of the anatomy, physiology, histology and microbiology of the digestive tract, the three-week-old piglet appears to be an appropriate model for studying aspects of protein digestion in the three-month-old human infant. To date, however, there has been no direct comparison of protein digestion in the two species. Although it would appear reasonable to assume that the piglet is an appropriate model for the human infant, further validation work is required, preferably involving direct comparison of the two species, before the piglet model can be used routinely.

## 1.8 OVERALL CONCLUSION AND INFERENCES FROM THE REVIEW OF LITERATURE

It would appear, from a review of the literature, that the gross amino acid composition of human milk may not be the best standard on which to base estimates of the amino acid requirements of infants. The presence of biologically active proteins, such as secretory immunoglobulin A and lactoferrin, in large quantities in human milk, and their apparent resistance to digestion in the infant's gastrointestinal tract, suggest that the amino acid composition of human milk needs to be corrected for digestibility.

Prior to this correction, however, the gross amino acid composition must be accurately determined taking into account the loss of amino acids during acid hydrolysis. Digestibility coefficients for each individual amino acid rather than a single correction factor for crude protein should be used, preferably determined at the terminal ileum and corrected for the endogenous excretion of amino acids. Difficulties involved with ileal sampling in human infants dictates the need for a model animal. The piglet would appear to be an appropriate model animal for studying aspects of protein digestion in the human infant. A direct comparison of protein digestion in the human infant and piglet would be useful, however, to support the indirect comparisons that have already been made. Finally, before the ileal digestibility of the amino acids in human milk can be determined using the piglet, the assumption that amino acids are not absorbed in nutritionally significant amounts from the large intestine of the piglet needs to be investigated.

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# CHAPTER 2

# THE THREE-WEEK-OLD PIGLET AS A MODEL ANIMAL FOR STUDYING PROTEIN DIGESTION IN HUMAN INFANTS

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Although the piglet has been suggested as an appropriate model for studying aspects of digestion in the human infant, no direct comparisons of digestion in the human infant and piglet have been made. Therefore, the aim of the study reported in this chapter was to directly compare, under controlled experimental conditions, the infant's and piglet's capacity to digest protein over the entire gastrointestinal tract.

## 2.1 ABSTRACT

The piglet was evaluated as a model animal for studying the digestion of high-quality proteins in human infants. Three-week-old male piglets (n=6) and three-month-old male human infants (n=6) were fed a bovine-milk-based formula over a 17 day experimental period comprising 7 days adaptation followed by a 10 day faecal collection period. The piglets and infants were given 345g liquid formula/kg body weight/day and 170g liquid formula/kg body weight/day, respectively, which equated to similar dry matter intakes per unit stomach volume (0.923g dry matter/$cm^3$/day). Both the piglets and infants were individually bottle-fed the reconstituted milk-formula (12.2% dry matter) at similar meal frequencies. Small but statistically significant differences ($P<0.01$) were found for the apparent faecal digestibility (mean ± overall SE) of dietary dry matter (98.8% vs 97.4% ± 0.13%), organic matter (99.0% vs 97.7% ± 0.12%) and total nitrogen (97.5% vs 94.5% ± 0.36%) between the piglets and infants. The faecal digestibilities for most of the amino acids were not significantly different ($P>0.05$) between the species. The digestion of protein appeared to be similar in the two species. The study provides support for using the piglet as a model animal for studying protein digestion in human infants.

## 2.2 INTRODUCTION

Some aspects of nutritional research are difficult or impossible to conduct with humans due to the invasive nature of the techniques employed. Model animals are being used increasingly to circumvent such difficulties, at the same time introducing greater flexibility and control over experimental procedures.

Several species of animal have been used in human nutrition research (Mitruka *et al.* 1976) and while non-human primates may seem an obvious choice, difficulties inherent in the handling and maintenance of these animals limits their usefulness (Mitruka *et al.* 1976). The pig has been used extensively in human nutrition research (Miller and Ullrey, 1987) and has been suggested as an appropriate model for studying the digestion and absorption of nutrients in human infants (Glauser, 1966).

The piglet appears to be particularly suitable for studying aspects of protein digestion in human infants (Rowan and Moughan, 1989; Moughan *et al.* 1992). In a recent comparative review of the digestive process in milk-fed piglets and human infants, Moughan *et al.* (1992) concluded that the digestive tracts of piglets and human infants are very similar in respect to anatomy, histology and aspects of digestive physiology. At birth there appears to be quite large differences between the species for the activities of the proteolytic enzyme, trypsin, and small-intestinal dipeptidases, reflecting a greater degree of development in the human at birth. However, if comparison is made between the two species at the more physiologically comparable stage of peak lactation in the mother (approximately 3 weeks in the piglet and 3 months in the infant), the major enzymes involved in the digestion of milk protein and the other milk components have similar activities in piglets and human infants. There is, however, one significant difference between the two species. At the comparable developmental stage of peak lactation in the mother, although the body weights of the two species are similar, the gut capacity of the piglet is around double that of the human infant. This difference needs to be considered when using the piglet as a model animal.

While indirect comparison of the digestive process in piglets and human infants highlights similarities, there has been no direct comparison of digestion made in the two species. The present study, a controlled comparison of the digestion of a bovine-milk-based protein by piglets and human infants, provides direct evidence as to the suitability of the piglet as a model animal for studying the digestion of high-quality proteins in humans.

## 2.3 EXPERIMENTAL

The human and animal research protocols were approved by the Massey University Human Ethics and Massey University Animal Ethics Committees, respectively.

### 2.3.1 **Subjects**

*Human Infants*

Six full-term (mean birth weight ± SE, 3.8 ± 0.12 kg) male infants with an average age of 86 days (mean body weight ± SE, 7.2 ± 0.27 kg) participated in the study. The infants had all been fully bottle-fed bovine-milk-based infant formulas from a minimum of one month after birth, and did not receive any supplementary food. Written permission for the infants to take part in the study was obtained from the parents of each infant. The parents were fully informed of the experimental procedure with all aspects of the study being carefully demonstrated. The experimental work involving the infants was subsequently conducted by the parents in their own homes, but with daily technical supervision to ensure compliance with the experimental procedures.

*Piglets*

Twelve Landrace X Large White male piglets (average age 7 days; mean body weight ± SE, 3.0 ± 0.17 kg) were selected at random from a group of piglets originating from five different litters of pigs (Pig Research Unit, Massey University). The piglets were penned individually in moulded-plastic metabolism cages. The animals were kept in a temperature controlled room maintained at 30 ± 1°C with a constant 15 hour light : 9 hour dark cycle.

### 2.3.2 **Diet and Feeding**

The ingredient and nutrient compositions of the commercially prepared infant formula, SMA Gold Cap (John Wyeth & Brother (NZ) Ltd, Auckland, New Zealand) are given in Table 2.1. The dry milk-formula was mixed with boiled distilled water (14g dry matter (DM)/100ml water) daily and kept refrigerated (4°C). The daily allowances were set at 169.7g liquid formula/kg body weight/day for the infants and 345.1g liquid formula/kg body weight/day for the piglets. These daily allowances corresponded to a similar level of food intake on a gut capacity basis (7.6g liquid formula/$cm^3$ of stomach volume) for the infants and piglets, assuming approximate stomach volumes of 22.4$cm^3$/kg body weight and 45.6$cm^3$/kg body weight for the infants and piglets, respectively (Moughan *et al.* 1992).

**Table 2.1.**

*Ingredient and nutrient composition* of a commercially prepared bovine-milk-based infant formula+*

| **Ingredient** | **g/100g#** |
|---|---|
| Skim Milk Powder | 2.02 |
| Demineralised Whey Powder | 3.38 |
| Lactose | 3.21 |
| Palm Oil | 1.13 |
| Coconut Oil | 1.13 |
| Soybean Oil | 1.13 |
| Minerals/Vitamins§ | 0.21 |
| **Nutrients** | **g/100g#** |
| Crude Protein¶ | 1.49 |
| Fat | 3.49 |
| Carbohydrate (lactose) | 6.97 |
| Ash | 0.26 |
| Energy (MJ GE/100g) | 26.58 |
| Essential Amino Acids (mg/100g) | |
| Lysine | 115.16 |
| Histidine | 39.36 |
| Methionine | 48.25 |
| Threonine | 74.61 |
| Tryptophan | 22.33 |
| Isoleucine | 90.60 |
| Leucine | 157.06 |
| Phenylalanine | 66.50 |
| Valine | 92.24 |

* The amino acid contents were determined except for that of cysteine and tryptophan; all other values are taken from the manufacturer's specifications.

\+ SMA Gold Cap (John Wyeth & Brother (NZ) Ltd, Auckland, New Zealand).

\# g/100g liquid formula, reconstituted at 12.2% Dry Matter.

§ Minerals and vitamins added to supply: C, 43.31; P, 28.91; K, 57.83; Na, 15.49; Cl, 39.28; Mg, 4.68; Fe, 1.02; Zn, 0.52; vitamin E, 0.96; ascorbic acid, 5.71; nicotinic acid, 1.02; pantothenic acid, 0.22; and choline, 10.32 (mg/100 g liquid formula); Mn, 15.86; Cu, 48.19; I, 6.22; Phylloquinone, 5.50; thiamin, 68.81; riboflavin, 103.21; pyridoxine, 43.31; cyanocobalamin, 0.13; retinol, 0.62; cholecalciferol, 1.02; pteroylmonoglutamic acid, 5.16; and biotin, 1.51 (µg/100 g liquid formula).

¶ 60% Whey protein:40% Casein protein.

The liquid milk-formula was warmed (35°C) prior to feeding. The amount of formula allocated daily to each infant and piglet was divided into equal portions. The infants were given their daily allocation of formula at times decided by their parents, with the number of feedings ranging from 5-8/day over all six infants. The piglets received their daily allocation of formula in seven portions every 2½ hours throughout the day from 0630 h to 2130 h.

### 2.3.3 **Experimental Procedure**

The infants were given their milk formula quantitatively by bottle-feeding, for a total of 17 days. The daily allocation of formula for each infant was made in a single batch. The parents divided this batch into a set number of meals. Any spillage together with milk-formula posseted by the infants was collected, measured and intakes corrected. Allowances were set at the beginning of the 17 day period without any further adjustment for change in bodyweight. Unsettled infants were given boiled water instead of extra formula. A seven day preliminary period was followed by a 10 day total faeces collection period.

For the entire 10 day faecal collection period the infants were fitted with disposable diapers specifically designed for boys (Pampers Nappies for Boys, Proctor and Gamble Ltd, Auckland, New Zealand). Three pre-weighed disposable diaper liners (Treasures Nappy Liners, Treasures Ltd, Auckland, New Zealand) were layered over the middle and back of each diaper to collect the faeces and absorb any softer faecal material. The parents were instructed to place the infant's penis upwards to direct the urine to the absorptive material present in the upper diaper. The edges of the three diaper liners were folded and placed between the infant's penis and scrotum to help prevent urine contamination of the faeces. The diaper was changed as soon as possible after defecation. The infant's skin was wiped with additional pre-weighed diaper liners. The soiled diaper and all liners were placed in a plastic bag and held on ice in an insulated container while awaiting collection. The material was collected within one hour of defecation and following collection, the faecal matter was scraped from the diaper liners and frozen (-20°C). The liners were freeze-dried, weighed and the amount of faecal DM remaining on the liner was determined by difference in weight.

In the six days following removal from the sow, the piglets were trained to drink their milk from bottles with soft rubber teats attached. During the study the piglets were individually hand-fed their formula and any liquid remaining after a meal

was weighed before being discarded. During most feedings there was minimal spillage of formula. A plastic container was held under the head of the piglet during each feeding to collect any spillage, which was subsequently weighed and deducted from the total intake for that meal. The piglets were given their formula at the designated allowance for three days, after which their allowances were adjusted for bodyweight and a steady intake maintained for a further four days. The piglets then underwent a 10-day total faeces collection period. The piglet's milk intakes were again adjusted on day 5 of the latter period. This was done to maintain a relatively constant milk-formula intake on a body weight basis, for the rapidly growing piglets.

At the beginning of the 17 day study, each piglet was shaved around the anal and tail region, and karaya base plates (Combihesive C321, E.R. Squibb & Sons Ltd, Auckland, New Zealand) designed for the attachment of human ostomy bags were adhered to the skin with adhesive tape (Elastoplast, Smith and Nephew Ltd, Auckland, New Zealand) so that the 32 mm opening in each plate was directly over the anus. A 100 x 100 mm pre-weighed plastic ostomy bag (Combihesive C329, E.R. Squibb & Sons Ltd, Auckland, New Zealand) was attached to the base plate. By using the ostomy bag it was possible to ensure total collection of uncontaminated faeces. Faeces present in the ostomy bags were usually removed within one hour of defecation and stored frozen (-20°C). After emptying, the soiled ostomy bags were freeze-dried, weighed and any remaining faecal DM determined by difference in weight.

### 2.3.4 **Chemical analysis**

A representative sample of the formula and the total faeces from the 10 day collection were freeze-dried. The faeces samples from each infant or piglet were thoroughly mixed and a subsample was taken for chemical analysis. Samples were analysed in duplicate for DM, organic matter, total nitrogen (N) and amino acids. For DM, organic matter, N and amino acids, the overall mean differences between duplicates within samples (expressed as a proportion of the mean), were 1.1%, 1.6%, 2.4% and 4.8%, respectively.

The DM and organic matter contents of the diet and faeces were determined after drying samples in a forced-air oven at 60°C until a constant weight was achieved for DM, followed by ashing in a furnace at 500°C for 16 hours to determine organic matter. Total N was determined on both the dietary and faecal samples by the Kjeldahl

method (Association of Official Analytical Chemists[1], 1980). Standard samples of analytical grade ammonium ferrous sulphate were used to test the accuracy of the N analysis. The recovery value for total N was 99.8%. The amino acid content of the diet and faeces were determined on 5mg freeze-dried samples, hydrolysed in 6M glass-distilled HCl (containing 1% phenol w/v) at 110°C for 24 hours, in sealed evacuated tubes. Amino acids were determined using ion exchange chromatography with ninhydrin detection on a Pharmacia Alpha Plus 4151 Series 2 Autoanalyser (Cambridge, London). Free amino acid molecular weights were used to convert moles to mg of amino acid. Using this method for amino acid analysis, methionine recovery is the same as that achieved under oxidative conditions (Rowan, 1989), thus, methionine values were recorded. Cysteine and tryptophan, being destroyed under acid hydrolysis, were not determined.

To confirm that urinary contamination of the infants' faeces had not occurred, the creatinine content of the faeces was determined. Duplicate 100mg samples of freeze-dried faeces were extracted with 5 ml of distilled, de-ionised water. The mixture was filtered (45 micron) and the filtrate analysed for creatinine colorimetrically (Larsen, 1972) on a Cobas Fara II autoanalyser (Hoffman-La Roche, Basel, Switzerland).

### 2.3.5 **Data Analysis**

Apparent nutrient digestibilities (%) were calculated from the total food intakes and faecal outputs of nutrients, pertaining to the 10 day experimental period, using the following equation:

$$\text{Digestibility (\%)} = \frac{\text{total nutrient intake} - \text{total nutrient faecal output}}{\text{total nutrient intake}} \times \frac{100}{1}$$

From prior observation (A.J. Darragh, unpublished) with three-week-old piglets fed highly digestible diets, it was expected that some individual animals would defecate infrequently, possibly leading to error in the determination of nutrient digestibility. Therefore defecation rates of both the infants and piglets were recorded. An *a priori* decision was made to calculate the digestibility coefficients based on the six piglets with defecation rates closest to those shown by the infants.

The digestibility of amino acid N was calculated using the sum of the N

[1]*Here after AOAC (1980)*

contributed from the amino acids (based on molecular weight) in the diet and faeces.

The mean digestibilities for DM, organic matter, N, amino acid N and the amino acids were compared using a simple one-way analysis of variance (SAS, 1985), for each component separately.

## 2.4 RESULTS

One piglet developed diarrhoea and was excluded from the study. The remaining piglets and the infants appeared healthy and readily consumed their set daily allowances of milk-formula. The ages and weights of the infants participating in the study are given in Table 2.2. The mean (± SE) growth rate for the infants (33 ± 2.0 g/day) was considered normal for 3-6 month-old infants. The mean (± SE) growth rate for the piglets of 122 ± 9.3 g/day (n=12), while lower than that expected for piglets suckled by the sow, was considered acceptable.

**Table 2.2.**

*The ages and weights of the infants participating in a 17 day nitrogen balance study*

| Infant | Birth Weight (kg) | Age* (days) | Weight (kg) | |
|---|---|---|---|---|
| | | | Day 1 | Day 17 |
| R.B | 4.16 | 70 | 6.8 | 7.3 |
| A.M | 3.73 | 83 | 6.3 | 6.8 |
| S.G | 3.35 | 84 | 6.9 | 7.6 |
| J.N | 3.64 | 88 | 6.7 | 7.3 |
| M.B | 3.64 | 103 | 6.4 | 6.9 |
| M.L | 4.05 | 86 | 7.4 | 8.0 |
| **Average** | 3.76 | 86 | 6.8 | 7.3 |

* Age at the beginning of the collection period, ie. Day 8.

The mean (± SE) actual daily milk-formula intakes during the 10 day experimental period were 138.5 ± 6.72g liquid formula (17.0 ± 0.82g DM)/kg body weight/day for the infants and 293.4 ± 10.08g liquid formula (36.0 ± 1.23g DM)/kg body weight/day for the piglets. These equated to daily intakes, on an assumed stomach volume basis of 6.2g liquid formula/$cm^3$ and 6.4g liquid formula/$cm^3$ for the

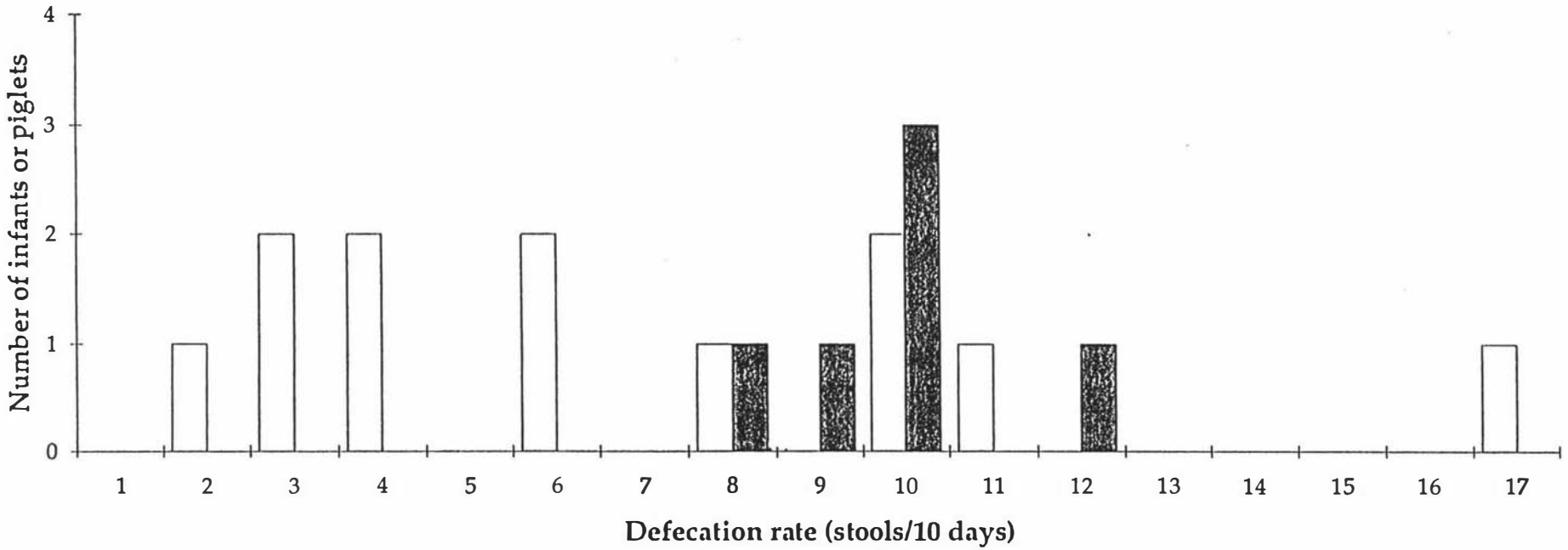


**Figure 2.1.**
The number of discrete defecations over a 10 day period for three-week-old piglets (clear) and three-month-old human infants (shaded), fed a bovine milk-based infant formula.

infants and piglets, respectively.

Careful visual examination of the diapers from each infant showed that in all instances the total faecal excretion had been collected on the liners with no faecal matter reaching the actual diaper and that most of the urine had been absorbed in the front of the diaper. A plot of the frequency of defecation observed for both the infants and piglets in the study is given in Figure 2.1. The infants had an average defecation rate of 1 stool per day. Defecation frequency was more variable for the piglets with a range of 2-11 defecations per animal over the 10 days. The DM and N digestibility coefficients (mean ± SE), calculated for all 11 piglets in the study were 99.1 ± 0.19% and 98.2 ± 0.45% for DM and N, respectively. However, the statistical comparisons given in Tables 2.3 and 2.4 are restricted to the six piglets with defecation rates (average of 9 stools passed over the 10 day period; range, 6-11 stools) closest to those observed in the infants. The stools of the infants contained significantly less DM ($P<0.05$)(mean ± SE; 27.6 ± 0.004 %) than the piglet's faeces (mean ± SE; 40.5 ± 0.01%).

The small differences found between the infants and piglets for the mean apparent faecal digestibility of DM, organic matter and N were statistically significant ($P<0.01$) while the mean apparent faecal amino acid N digestibilities were not significantly different (Table 2.3).

**Table 2.3.**

*Apparent faecal digestibilities (%) of gross nutrients in a commercially prepared bovine-milk based infant formula[+] fed to three-month-old human infants and three-week-old piglets*

(Mean values with the overal standard error for six human infants and six piglets)

| | Human Infant | Piglet | Overall SE | Statistical Significance[#] |
|---|---|---|---|---|
| Dry Matter | 97.4 | 98.8 | 0.13 | ** |
| Organic Matter | 97.7 | 99.0 | 0.12 | ** |
| Total Nitrogen | 94.5 | 97.5 | 0.36 | ** |
| Amino Acid Nitrogen[§] | 96.2 | 97.1 | 0.34 | NS |

[+] SMA Gold Cap (John Wyeth & Brother (NZ) Ltd, Auckland, New Zealand).

[#] NS = non-significant, $P>0.05$; ** = $P<0.01$.

[§] Calculated based on the sum of the N contributed by each amino acid.

The mean apparent amino acid digestibilities are given in Table 2.4. There were no significant differences in digestibility for most of the essential amino acids, with the exception of threonine. Likewise, the non-essential amino acids appeared to be digested to a similar extent by the infants and piglets, with the exception of glutamic acid, proline and serine.

The mean (± SE) concentration of creatinine measured in the infant's faeces was 0.23 (± 0.031) mg/g faecal DM. The range for the faecal creatinine was 0.14-0.36mg of creatinine/g faecal DM.

**Table 2.4.**

*Apparent faecal digestibilities (%) of amino acids in a commercially prepared bovine-milk based infant formula[+] fed to three-month-old human infants and three-week-old piglets*

(Mean values with the overal standard error for six human infants and six piglets)

| | Human Infant | Piglet | Overall SE | Statistical Significance[#] |
|---|---|---|---|---|
| Lysine | 96.6 | 97.6 | 0.65 | NS |
| Histidine | 96.7 | 97.5 | 0.29 | NS |
| Arginine | 94.8 | 95.5 | 0.56 | NS |
| Aspartic Acid | 96.4 | 96.9 | 0.36 | NS |
| Threonine | 96.1 | 97.4 | 0.34 | * |
| Serine | 96.1 | 97.5 | 0.33 | * |
| Glutamic Acid | 97.6 | 98.3 | 0.21 | * |
| Proline | 97.9 | 98.7 | 0.17 | ** |
| Glycine | 91.1 | 93.2 | 0.76 | NS |
| Alanine | 93.3 | 94.8 | 0.60 | NS |
| Valine | 96.8 | 96.8 | 0.37 | NS |
| Methionine | 97.3 | 97.0 | 0.38 | NS |
| Isoleucine | 97.3 | 97.2 | 0.31 | NS |
| Leucine | 97.5 | 97.5 | 0.28 | NS |
| Tyrosine | 96.0 | 96.2 | 0.44 | NS |
| Phenylalanine | 95.3 | 96.3 | 0.41 | NS |

[+] SMA Gold Cap (John Wyeth & Brother (NZ) Ltd, Auckland, New Zealand).

[#] NS = non-significant, P>0.05; * = P<0.05; ** = P<0.01.

## 2.5 DISCUSSION

The aim of the present study was to evaluate the piglet as a model animal for studying the digestion of protein in high-quality foods for human infants. Accordingly, a direct comparison of N and amino acid digestibility was made between the piglet and human infant.

There is the potential in balance studies, such as this one, for the digestibility of a nutrient to be overestimated due to overestimation of intake and, or the underestimation of faecal output. This is particularly so, for the infant where it is not possible to exercise the same degree of control as for the young pig. In the design and conduct of this study careful attention was given to these two areas. As the infants involved in the present work were studied at home rather than in the controlled environment of a metabolic ward, the potential for methodological error was increased. However, the parents involved in the study were well-instructed and closely supervised, and it is unlikely that there were major methodological shortcomings. One advantage of the present study is that the infants were studied in their normal environment, rather than in a metabolic ward.

It was important that urine contamination of the faeces was avoided. If urinary contamination had occurred, the total N digestibility would have been underestimated. The amino acid N and amino acid digestibility values would not have been greatly influenced by urinary contamination, however, as the proportion of amino acid N in urine is relatively small (Geigy Sceintific Tables, 1970). The infant's faeces were collected from a diaper and inspection of each diaper, post-collection, revealed that most of the urine was retained within the front portion of the diaper where extra absorptive material was present. Although care was exercised, there was still the possibility that some urinary contamination occurred. Creatinine, which is excreted almost exclusively in urine, was thus measured in the present study to confirm that urinary contamination of the infant's faeces had not occurred. In milk-fed infants approximately 12mg of urinary creatinine/kg body weight is excreted daily (Geigy Sceintific Tables, 1970). Therefore, a 7kg infant would excrete approximately 84mg of urinary creatinine/day. Creatinine measured in the present study, averaged 0.23mg creatinine/g faecal DM or 0.73mg creatinine/day for the infants. Assuming that all of the creatinine measured in the infants' faeces was of urinary origin, this suggests a urinary contamination of less than 1%.

The digestibilities determined in the present study were based on total nutrient

intakes and the total excretion of nutrients in the faeces. This relies on the assumption that the faecal matter collected directly related to nutrient intake. As the infants were maintained on a steady food intake for 17 days, the faeces collected over the final 10 days should have been directly related to food intake. For the rapidly growing piglets, however, and with the need to maintain a steady intake of food DM proportional to body weight, it was necessary to increase the piglet's food intake at the mid-point of faecal collection. Given that a 40-45 hour rate of passage of dietary DM has been observed in piglets fed a bovine milk-based diet (Wilson and Leibholz, 1981a), there may have been a discrepancy between the faecal output and average nutrient intake, resulting in higher nutrient digestibilities. However, when the DM digestibility was re-calculated conservatively, using a 10 day total food intake based on the average daily intake of formula by piglets during the first 5 days of the collection period only, there was no significant difference ($P>0.05$) in DM digestibility. Therefore, it was concluded that the digestibility values based on the total collection did not introduce a serious bias.

The apparent faecal digestibilities of N for piglets in the present study were similar to values in the literature for the digestibility of dietary N in piglets fed a milk-formula (Wilson and Leibholz, 1981b; Moughan *et al.* 1990). The mean N digestibility for the infants in the present study was higher than the range of 87-90% for N digestibility previously reported in the literature for formula-fed human infants (Fomon, 1961; Slater, 1961; Widdowson, 1965; Southgate and Barrett, 1966; Cordano *et al.* 1988). This may have been related to the application of the balance technique in an outpatient setting, although the low between-subject variation observed in the present study (CV% of 0.55% and 1.16% for N digestibility in the infants and piglets, respectively) indicates that a high degree of precision was obtained. The infants in the present study were older that those in several of the previous studies, which may also have contributed to the observed differences in N digestibility. Further, the longer duration of the collection period in the present study may have contributed to the difference. This is supported by evidence from one study (Prentice *et al.* 1987) where a 7 day balance study was conducted in an outpatient setting, using diaper-collection of faeces from 3-month-old breast-fed infants. In this study the apparent faecal digestibility of N in the human milk was 95%, similar to the digestibility value reported in the present study.

When the apparent faecal digestibilities for DM, organic matter, and total N

were compared, the piglet appeared to digest food to a greater extent than did the infant. Although statistically significant, the actual differences in the digestibility of dietary DM, organic matter and total N were small and of little practical significance. The statistically significant species difference for total N digestibility was not reflected in the amino acid N digestibility comparison and the apparent faecal amino acid digestibilities were also similar between the piglets and infants. It appears, therefore, that the digestion of protein is similar in the three-month-old human infant and the three-week-old piglet, at least for highly-digestible milk proteins.

The faecal measure of digestibility has shortcomings (Sauer and Ozimek, 1986), however, and it would be preferable to compare the species by determining protein digestibility at the end of the small intestine. The latter comparison is difficult with the human infant. However, where ileal amino acid digestibility has been compared (Rowan *et al.* 1994) in growing pigs and ileostomised adult humans, close inter-species agreement was found.

The present study has established that the apparent digestibility of amino acids, determined over the entire digestive tract, is generally similar for human infants and piglets receiving a high-quality milk protein based diet. This provides support for using the piglet as a model animal for studying protein digestion in human infants. Further work needs to be conducted, however, using a wider range of dietary protein sources.

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# CHAPTER 3

# ABSORPTION OF LYSINE AND METHIONINE FROM THE PROXIMAL COLON OF THE PIGLET

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The results reported in chapter 2 provide support for the piglet as a model animal for studying aspects of protein digestion in the human infant. Application of the piglet model to determine protein digestion would most likely involve the use of an ileal digestibility assay which relies on the assumption that amino acids are not absorbed in nutritionally significant amounts from the large intestine. There is *in vitro* evidence, however, to suggest that this latter assumption may not be valid in the neonatal piglet. Therefore, the following study investigated whether lysine and methionine are absorbed in nutritionally significant amounts from the large intestine of the piglet.

## 3.1 ABSTRACT

The present study aimed to determine whether lysine and/or methionine are absorbed in nutritionally significant amounts from the proximal colon of milk-formula-fed piglets (15-32 day old; 2.0-7.4 kg liveweight). Piglets, surgically prepared with simple catheters which allowed infusion into the proximal colon, were randomly allocated to one of two milk-formula diets which were either 40% deficient in lysine (L- diet) or 60% deficient in methionine and 40% deficient in cysteine (S- diet), yet balanced for all other amino acids. The piglets were individually bottle-fed the milk-formula diets seven times daily at 2 h intervals between 08.00 and 20.00 hours. Physiological saline (9 g NaCl/l) or an isotonic solution containing the deficient amino acid was infused via the catheter at each feeding. The experimental procedure followed a cross-over design. Total daily excretions of urinary urea and total N were determined. There were no significant differences ($P>0.05$) in urinary N metabolite excretion for piglets infused with amino acids compared with those infused with saline. Lysine and methionine do not appear to be absorbed in nutritionally significant amounts from the proximal colon of the milk-fed piglet.

## 3.2 INTRODUCTION

Determination of amino acid digestibility at the terminal ileum of the pig is generally considered to be more accurate than the traditional faecal method (Low, 1980; Moughan and Smith, 1982; Sauer and Ozimek, 1986). Differences between faecal and ileal amino acid digestibilities have been found with growing pigs (Zebrowska, 1978; Jorgensen and Sauer, 1982) and milk-fed piglets (Moughan *et al.* 1990). The ileal method, while apparently superior to the faecal digestibility technique, relies on the assumption that amino acids are not absorbed in nutritionally significant amounts from the large intestine. N absorbed from the large intestine is thought to be in the form of ammonia together with a small amount of amines and amides, which are ultimately excreted mainly as urinary urea (Hoover and Heitmann, 1975; Zebrowska, 1982; Just, 1983; McNiel, 1988).

Whereas several studies have shown that amino acids are not absorbed in significant amounts from the large intestine of growing pigs (Zebrowska, 1973,1975,1978; Just *et al.* 1981; Schmitz *et al.* 1991), there is some evidence for amino acid absorption from the large intestine of the piglet during the first week after birth. James and Smith (1976) found that at birth the piglet proximal colon has a structure which is similar to that of the small intestine and speculated that absorption of amino acids in the hindgut of young animals may be of physiological importance. Furthermore, results of several *in vitro* studies have shown that methionine is absorbed into the proximal colonic epithelium of new-born piglets (James and Smith, 1976; Smith and James, 1976; Jarvis *et al.* 1977). While there are no published quantitative data on the *in vivo* absorption of amino acids in neonatal piglets, Heine *et al.* (1987) demonstrated absorption and retention of protein $^{15}N$ in the colon of human infants. In the latter study, however, it is likely that the protein $^{15}N$ was degraded by intestinal bacteria and absorbed as labelled ammonia.

The present study aimed to determine whether the dietary essential amino acids, lysine and methionine, are absorbed from the proximal colon of the milk-formula-fed piglet. The study examined the effect, on urinary N metabolite excretion, of infusions of free lysine or methionine into the proximal colon of piglets fed with liquid milk-formula diets which were either 40% deficient in lysine or 50% deficient in the sulphur amino acids.

## 3.3 EXPERIMENTAL

All aspects of this study were approved by the Massey University Animal Ethics Committee.

### 3.3.1 **Animals and housing**

Twenty-one 6-day-old entire male Landrace X Large White piglets were selected at random from six litters of pigs at the Pig Research Unit, Massey University. Sixteen piglets were randomly chosen for the implantation of colonic catheters and five piglets remained unoperated. The piglets were individually and randomly penned in moulded-plastic metabolism cages which were designed to allow complete collection of urine. The animals were kept in a temperature-controlled room maintained at 30 ± 1°C.

### 3.3.2 **Surgical preparation**

Anaesthesia was induced and maintained throughout surgery with halothane (Fluothane; Imperial Chemical Industries Ltd) which was inhaled through a mask, using concentrations of 5% in $O_2$ for induction and 2-3% in $O_2$ for maintenance. Each piglet was placed in left lateral recumbency and a 30 - 40 mm dorsal-ventral incision was made in the flank midway between the last rib and the pelvis. The caecum was located and exteriorized; a purse-string suture was inserted in the caecal wall as close as possible to the ileo-caecal junction. The catheter was inserted via an incision made through the wall of the caecum within the area of the purse-string suture. The catheter, which consisted of a 300 mm length of silastic medical-grade tubing (3.2 mm OD, 1.6 mm ID, dead space volume 1 ml; Dow Corning Corporation, Midland, MI, USA), had two silastic cuffs placed 3 mm apart, secured 50 mm from one end of the tubing with silastic, medical-grade adhesive (Dow Corning Corporation). After the catheter was inserted into the caecum the cuffs were positioned on either side of the caecal wall and the purse-string suture tied securely around the catheter between the two cuffs. The tip of the catheter was positioned in the proximal large intestine so that it was distal to the ileo-caecal junction. A second purse-string suture was inserted to secure the catheter which was subsequently laid along, and sutured to, the external caecal wall. The external tip of the catheter was sealed with a metal pin. Using a specially designed trochar and cannula, the catheter was tunnelled through the peritoneum and muscle layers, and then subcutaneously to the dorsum of the piglet where it was externalized in the mid-lumbar region. A towelling pouch with a Velcro seal was attached to the dorsum of the piglet with elasticized adhesive tape. The 200

mm of exposed catheter was coiled and placed within the pouch. Surgical netting (Systenet; International Surgical Netting, Zurich, Switzerland) was placed over the body of the piglet to protect the pouch.

While under anaesthesia, each piglet was shaved around the anal and tail region, and karaya base plates (Combihesive C321; E.R. Squibb and Sons Limited, Auckland, New Zealand) designed for the attachment of human ostomy bags were affixed to the skin with elasticized adhesive tape so that the 32 mm opening in each plate was directly over the anus. A 100 x 100 mm plastic bag (Combihesive C329; E.R. Squibb and Sons Limited) was attached to the base plate. The five unoperated piglets, which were used to examine the possible effects of surgery on urinary N metabolite excretion, were also fitted with karaya base plates. By using the ostomy bag technique it was possible to ensure complete and separate collection of faeces from the piglets. The ostomy bags also prevented the practice of coprophagy.

### 3.3.3 **Diets**

The ingredient and nutrient compositions of the milk-formula diets used during the preliminary period (preliminary diet) and the experimental periods (L- diet and S- diet) are given in Tables 3.1 and 3.2. The diets used during the experimental periods were formulated to meet the amino acid requirements of piglets for growth except for either lysine (L-) or the sulphur amino acids (S-). The amino acid requirement was based on the composition of sow's milk at peak lactation (Agricultural Research Council, 1981). The diet deficient in lysine (L- diet) contained 60% of the required level of lysine, and the diet deficient in the sulphur amino acids (S- diet) contained 60% and 40% of the required levels of cysteine and methionine respectively, giving a diet that was 50% deficient in total sulphur amino acids. The estimated amino acid intakes of piglets fed either the L- or S- diets are compared with recommended daily amino acid intakes in Table 3.3.

During the last period of the experiment the diets fed to the catheterized piglets were modified (6.24 g lysine as lysine monohydrochloride/kg dry matter was added to the L- diet, and 3.93 g methionine/kg dry matter was added to the S- diet) to be balanced for lysine (L+ diet) and methionine (S+ diet).

The dry milk-formula diets were mixed with water (200g dry matter /kg liquid formula) daily and kept refrigerated at 4°C. The liquid milk-formula diets were warmed to 35°C before feeding.

**Table 3.1.**

*Composition of the milk-formula diets (g/kg oven-dry weight)*

| | Milk-formula diets | | |
|---|---|---|---|
| | Preliminary | L- | S- |
| Skimmed milk | 165.45 | 160.94 | 149.27 |
| Demineralised whey powder | 277.15 | 269.60 | 250.05 |
| Lactose | 263.00 | 255.84 | 237.28 |
| Palm oil | 92.40 | 89.89 | 83.36 |
| Coconut oil | 92.40 | 89.89 | 83.36 |
| Soya-bean oil | 92.40 | 89.89 | 83.36 |
| Minerals and vitamins* | 17.20 | 16.73 | 15.52 |
| Synthetic amino acids+ | | | |
| Lysine HCL | - | - | 17.70 |
| Methionine | - | 0.87 | - |
| Cysteine | - | 2.17 | 2.35 |
| Threonine | - | 1.65 | 8.18 |
| Tryptophan | - | 0.90 | 2.95 |
| Isoleucine | - | 2.07 | 8.43 |
| Leucine | - | 5.16 | 18.48 |
| Histidine | - | 2.93 | 7.12 |
| Phenylalanine | - | 2.98 | 9.02 |
| Tyrosine | - | 4.70 | 11.55 |
| Valine | - | 3.78 | 12.00 |

L-, milk-formula diet, 40% deficient in lysine; S-, milk-formula diet, 60% deficient in methionine and 40% deficient in cysteine.

* The mineral and vitamin content of preliminary diet (mg/kg oven-dry diet) Ca 3550, P 2370, K 4740, Na 1270, Cl 3220, Mg 384, Fe 84, Zn 43, vitamin E 79, ascorbic acid 468, nicotinic acid 84, pantothenic acid 18, choline 846; µg/kg oven-dry diet: Mn 130, Cu 395, I 51.0, vitamin K 45.1, thiamin 564, riboflavin 846; pyridoxine 355, cyanocobalamin 1.1, retinol 508, cholecalciferol 8.4, pteroylmonoglutamic acid 42.3, biotin 12.4.

The mineral and vitamin content of L- (mg/kg oven-dry diet): Ca 3450, P 2310, K 4610; Na 1240; Cl 3130, Mg 374; Fe 82, Zn 42, vitamin E 77, ascorbic acid 455, nicotinic acid 82, pantothenic acid 18, choline 823. µg/kg oven-dry diet: Mn 127, Cu 384, I 49.6, vitamin K 43.9; thiamin 549; riboflavin 823; pyridoxine 345, cyanocobalamin 1.1, retinol 494; cholecalciferol 8.2, pteroylmonoglutamic acid 41.1, biotin 12.1.

The mineral and vitamin content of S- (mg/kg oven-dry diet): Ca 3200, P 2140, K 4280; Na 1150; Cl 2910, Mg 346; Fe 76, Zn 39, vitamin E 71, ascorbic acid 422, nicotinic acid 76, pantothenic acid 16, choline 763. µg/kg oven-dry diet: Mn 117, Cu 356, I 46.0, vitamin K 40.7; thiamin 509; riboflavin 763; pyridoxine 320, cyanocobalamin 1.0, retinol 458; cholecalciferol 7.6, pteroylmonoglutamic acid 38.2, biotin 11.2.

\+ All synthetic amino acids were L-isomers.

**Table 3.2.**

*Nutrient composition of the experimental milk-formula diets (g/kg oven-dry diet)*

| | Diets | |
|---|---|---|
| | L- | S- |
| Crude protein | 146.30 | 208.25 |
| Fat | 277.94 | 257.77 |
| Lactose | 555.89 | 515.54 |
| Gross energy (MJ) | 21.09 | 19.56 |
| Ash | 19.85 | 18.41 |
| Lysine | 9.16 | 26.20 |
| Methionine | 3.49 | 2.43 |
| Cysteine | 3.20 | 3.31 |
| Threonine | 8.38 | 14.42 |
| Tryptophan | 2.68 | 4.60 |
| Isoleucine | 8.33 | 14.24 |
| Leucine | 14.52 | 27.16 |
| Histidine | 5.55 | 9.55 |
| Phenylalanine | 8.03 | 13.70 |
| Tyrosine | 9.00 | 15.54 |
| Valine | 10.79 | 18.51 |

L-, milk-formula diet, 40% deficient in lysine; S-, milk-formula diet, 60% deficient in methionine and 40% deficient in cysteine.

* Based on tabulated values.

### 3.3.4 Experimental procedure

In the 10 days following removal from the sow and before the commencement of the experimental period the piglets (average age 15 days, mean body weight (± SE) 2.85 ± 0.14 kg) were trained to drink the preliminary diet from bottles with soft rubber teats attached. Surgery was performed when the piglets were 10-11 days old, 4-5 days before the commencement of the experimental period. The piglets recovered rapidly from surgery and were given their daily allowances of milk-formula in equal amounts at 2 hourly intervals from 0800 h to 2000 h, i.e. seven feedings/d.

The sixteen catheterized piglets were allocated at random to either the L- diet or the S- diet (eight piglets/diet) and the five unoperated piglets were allocated to the S- diet (Table 3.4). The piglets received their respective milk-formula diets for two consecutive 6 day experimental periods (time-period 1 and time-period 2).

**Table 3.3.**

*Dietary essential amino acid intakes (g/day) of 4 kg piglets given either the L- or S- diets, compared with recommended (Agricultural Research Council, 1981) amino acid intakes for the milk-fed piglet.*

| | Amino acid intake | | |
|---|---|---|---|
| | L- | S- | Recommended* |
| Lysine | 1.81 | 3.02 | 3.02 |
| Methionine and cysteine | 1.32 | 0.66 | 1.32 |
| Threonine | 1.66 | 1.66 | 1.66 |
| Tryptophan | 0.53 | 0.53 | 0.53 |
| Isoleucine | 1.64 | 1.64 | 1.64 |
| Leucine | 3.42 | 3.42 | 3.42 |
| Histidine | 1.10 | 1.10 | 1.10 |
| Phenylalanine and tyrosine | 3.37 | 3.37 | 3.37 |
| Valine | 2.14 | 2.14 | 2.14 |

L-, milk-formula diet, 40% deficient in lysine given to piglets at a rate of 240 g liquid-milk-formula diet/kg liveweight; S-, milk-formula diet, 60% deficient in methionine and 40% deficient in cysteine given to piglets at a rate of 130 g liquid milk-formula diet/kg liveweight.

* Recommended levels calculated based on the amino acid composition of mature sow's milk, and the expected intake of sow's milk by a 4 kg suckled piglet (Agricultural Research Council, 1981).

**Table 3.4.**

*The design of the experiment*

| Time period... | | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|---|
| Age (d)... | | 15-20 | | 21-26 | | 27-32 | |
| Diet and treatment... | | Diet | Infusion | Diet | Infusion | Diet | Infusion |
| Pigs: | *n* | | | | | | |
| Catheterised* | 4 | L- | Saline | L- | Lysine | L+ | Saline |
| Catheterised | 4 | L- | Lysine | L- | Saline | L+ | Saline |
| Catheterised | 4 | S- | Saline | S- | Methionine | S+ | Saline |
| Catheterised | 4 | S- | Methionine | S- | Saline | S+ | Saline |
| Unoperated | 5 | S- | -# | S- | - | S- | - |

L-, milk-formula, 40% deficient in lysine; S-, milk-formula diet, 60% deficient in methionine and 40% deficient in cysteine; saline, 9 g NaCl/l.

* Two piglets in this group did not complete the experiment because their catheters became dislodged during time-period 1.

\# Unoperated pigs were not infused.

The piglets were weighed accurately (to within 10 g) at the beginning of each time period and were given the respective diets at set levels of intake (240 g and 130 g of liquid milk-formula diet/kg liveweight per day for the L- and S- diets respectively) to give the planned levels of amino acid deficiency. During a final 6 day period (time-period 3) the catheterized piglets which had previously received the L- diet were fed on a diet supplemented with synthetic lysine monohydrochloride (L+ diet), and piglets that received the S- diet were fed on a diet supplemented with methionine (S+ diet). The five unoperated piglets received the S- diet during all three time periods.

During time-period 1, four randomly selected catheterized piglets on each diet received infusions of the deficient amino acid into the proximal large intestine, while the other four piglets received infusions of physiological saline (9 g NaCl/l). In time-period 2 the infusion regime was reversed. The amounts of amino acid infused were 60.2 mg lysine/kg liveweight per day (13.3% lysine fed/kg liveweight per day) and 20.4 mg of methionine/kg liveweight per day (12.4% methionine fed/kg liveweight per day) for piglets given the L- diet and S- diet respectively. These amounts were based on the assumption that 80% of amino acids in a milk-formula diet would be completely absorbed by the terminal ileum. Thus the amounts of amino acid infused into the proximal colon approximated the levels of these amino acids expected to be flowing into the hind gut of normal, suckled piglets. The total amount of each synthetic amino acid to be infused into each piglet daily was dissolved in 10 ml distilled water to which NaCl was added to provide a 300 mM isotonic solution. The daily infusate was divided into seven equal portions with each portion being warmed to 37°C and infused at each of the seven feedings. All amino acid infusions were followed by a 1 ml infusion of physiological saline (9 g NaCl/l) to fill the dead-space of the catheter. The catheterized piglets not receiving an amino acid infusion during each time-period, were infused with 2 ml of physiological saline during each feeding. In time-period 3, all catheterized piglets received 2 ml infusions of physiological saline at each feeding.

During the final 3 days of each 6 day time-period the urinary output of each piglet was collected three times daily (08.00, 12.00 and 18.00 hours). The 3 day adaptation between each collection period was considered to be sufficient (Brown and Cline, 1972*a,b*). The plastic metabolism cages were designed to ensure rapid collection of filtered urine into narrow-necked plastic bottles containing 1.8 M-$H_2SO_4$ (25 ml acid/l urine collected). The procedure for collection of urine included spraying the cage floor

and sides three times daily with distilled water to minimize the loss of urinary nitrogen. Representative subsamples of the daily urine outputs were frozen. At the end of each time-period the daily urine outputs for each piglet over the 3 day collection period were bulked, subsampled and stored at -20°C. The ostomy bags remained intact during the trial, and faeces were collected daily from the ostomy bags and discarded.

At the completion of the 18 day experimental period the eight piglets that had been given the S+ diet had free homoarginine (HA; L-homoarginine, Sigma Chemical Company) infused into the colon. HA is a synthetic analogue of lysine that does not occur naturally in the pig's digestive tract. The HA infusions (8.6 mg/kg liveweight per infusion) were given 1 hour before (1100 h) and at the 1200 h feeding. The piglets were killed with an overdose of sodium pentobarbitone (Pentobarb, 300 mg/ml; South Island Chemicals Ltd, Christchurch, New Zealand) administered by intraperitoneal injection 30 min after the second infusion of HA. The body cavity was opened and the ileo-caecal junction immediately located and clamped. For each piglet the digesta from the final 200 mm of ileum were flushed out with distilled water, collected and frozen for determination of HA to monitor possible backflow of digesta into the small intestine. At death the status and position of the catheter in the large intestine were noted.

### 3.3.5 **Chemical Analysis**

Urine was subjected to chemical determination in duplicate for total N, urea and creatinine. Total N was determined by the Kjeldahl method (Association of Official Analytical Chemists, 1980) and urea and creatinine were determined colorimetrically on a Cobas Fara II autoanalyser (Hoffman-La Roche, Basel, Switzerland) using the methods of Tiffany *et al.* (1972) and Larsen (1972), respectively.

Standard samples of Analar grade ammonium ferrous sulphate, urea and creatinine were used to test the accuracy of the respective analyses. Recovery values for total N, urea and creatinine were 99.9%, 99.7% and 100% respectively. The precision (intra-assay variation) of the urea and creatinine assays was determined by including six standard samples per run of sixty test samples. The intra-assay coefficient of variation was 2.0% and 1.1% for urea and creatinine respectively. The overall mean differences between duplicates within samples (expressed as a proportion of the mean), were 2.1%, 1.5% and 1.0% for total N, urea and creatinine respectively.

Urinary N metabolite excretions were calculated on the basis that urea contains

46.7% N and creatinine 37.2% N, on a molecular weight basis, and the daily excretions were expressed per unit of metabolic body weight ($kg^{0.75}$). Freeze-dried ileal digesta samples (7 mg) were acid-hydrolysed in 0.5 ml 6 M-HCL (+ 0.1% phenol) for 24 hours at 110° ± 2°C, and prepared for determination of HA on a Pharmacia LKB-Alpha plus amino acid autoanalyser (Cambridge, England).

### 3.3.6 **Data Analysis**

Treatment means were compared using a simple one-way analysis of variance. A paired *t* test was used to compare urinary N metabolite excretion rates for piglets given either the L- or S- diets in time-period 2, with the urinary metabolite excretion rates for the same piglets given the L+ or S+ diets respectively, during time-period 3.

## 3.4 RESULTS

Two piglets (L- diet) did not complete the experiment because their catheters became dislodged during time-period 1. All catheterized piglets resumed normal feed intakes within 12 hours of surgery and, together with the unoperated piglets, appeared healthy and readily consumed their set daily intakes of milk-formula throughout the study. At the beginning of time-periods 1, 2 and 3 and at the end of time-period 3, average piglet liveweights (mean ± SE) were 2.85 ± 0.140 kg, 3.35 ± 0.185 kg, 3.93 ± 0.484 kg and 4.73 ± 0.310 kg respectively, with a range of 2.0 - 7.4 kg throughout the experiment.

HA could not be detected in any of the samples of ileal digesta from the catheterized piglets colonically infused with this synthetic amino acid. Thus, there did not appear to have been any backflow of infused amino acids into the small intestine. Post-mortem examination of all catheterized piglets showed that their catheters remained in place and that the tips of the catheters were all situated distal to the ileo-caecal junction.

The mean daily urinary N metabolite excretion rates in time period 2 were used to assess whether there was any effect of surgery *per se*. The differences in N metabolite excretions between catheterized and unoperated piglets given the S- diet in time-period 2 (Fig. 3.1) were not significantly different ($P>0.05$). Thus, surgical intervention and the establishment of chronic catheters in the large intestine did not appear to have any effect on N metabolism.

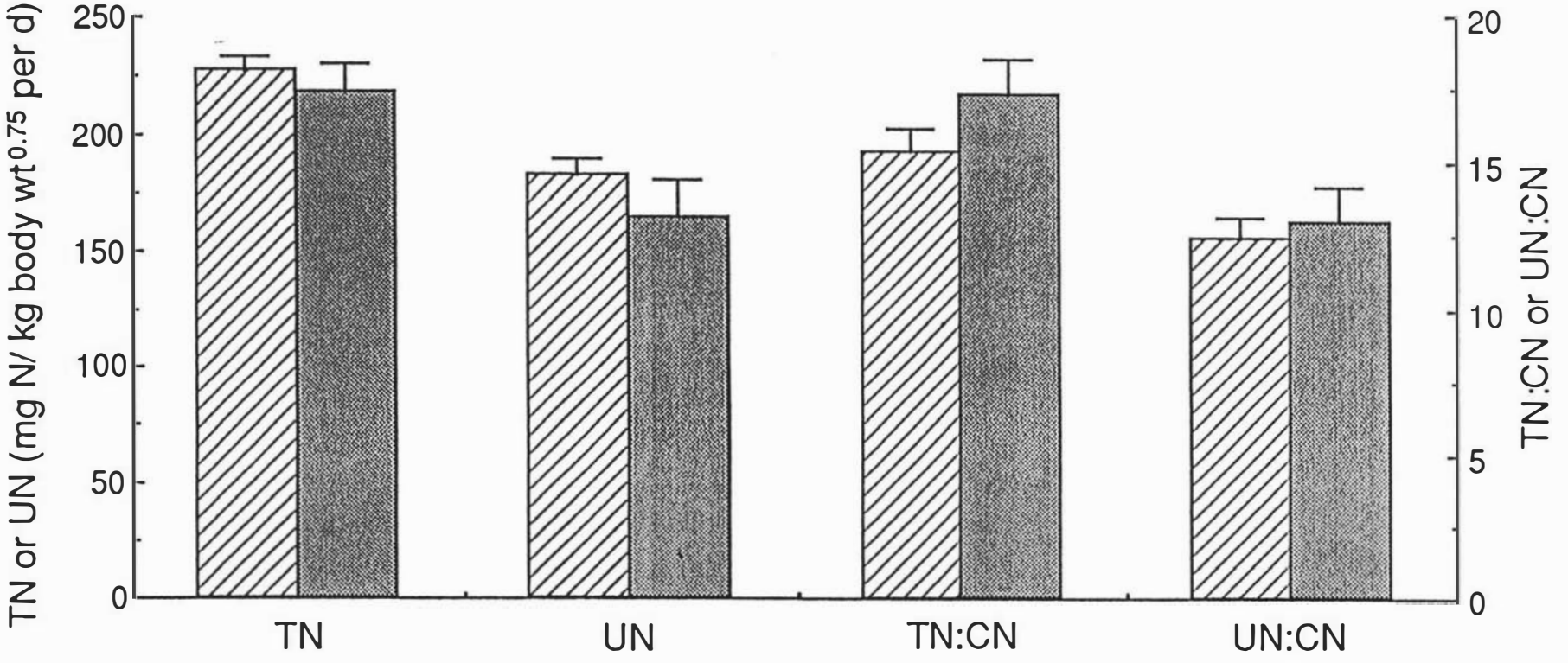


**Figure 3.1.**
Comparison of the mean daily urinary excretion of total N (TN) and urea-N (UN), and the ratios between TN and the mean daily urinary creatinine-N (CN) excretion (TN:CN), and between UN and CN (UN:CN) in time-period 2 for catheterized piglets (hatched; *n* 8) and unoperated piglets (shaded; *n* 5). All the piglets were fed with a milk-formula diet that was 60% deficient in methionine and 40% deficient in cysteine (S- diet). Values are means with their standard errors represented by vertical bars. Mean values for catheterized piglets were not significantly different from those for unoperated piglets: $P > 0.05$.

In all piglets there was no significant (P>0.05) effect of diet on daily urinary creatinine excretion either within or between time periods. The overall mean (± SE) for individual piglet's mean daily excretion of urinary creatinine was 38.0 ± 1.42 mg/kg$^{0.75}$ per day (range 29.0 - 49.5 mg creatinine/kg$^{0.75}$ per day). The overall mean for individual piglet's coefficient of variation for daily urinary creatinine excretion was 14.7% (range 9.8 - 22.1%).

Comparison of urinary total N and urea-N excretion for piglets fed either the L- or S- diets and infused with physiological saline or amino acids during time-periods 1 and 2 showed a significant (P<0.05) increase in excretion over time. As a result of this unexpected time effect, comparisons of urinary N metabolite excretion rates between piglets infused with either physiological saline or amino acids were restricted to within time periods (Tables 3.5 and 3.6).

**Table 3.5.**

*Mean daily urinary N metabolite excretion rates (mg/kg$^{0.75}$ per day) for piglets infused colonically with either physiological saline (9 g NaCl/l) or free lysine, while fed with a milk-formula diet 40% deficient in lysine (L- diet)*

(Mean values with their standard errors for two to four piglets)

| | Infusion | | | | Statistical Significance |
|---|---|---|---|---|---|
| | Saline | | Lysine | | |
| | Mean | SE | Mean | SE | |
| Time-period 1[+] | | | | | |
| Total N | 154.3 | 7.86 | 153.5 | 12.99 | NS |
| Urea-N | 89.5 | 6.47 | 101.8 | 8.29 | NS |
| Total N:creatinine-N | 10.4 | 1.16 | 11.3 | 0.21 | NS |
| Urea-N:creatinine-N | 6.0 | 0.54 | 7.5 | 0.20 | * |
| Time-period 2[+] | | | | | |
| Total N | 202.4 | 13.75 | 209.2 | 28.54 | NS |
| Urea-N | 142.1 | 11.98 | 158.0 | 5.14 | NS |
| Total N:creatinine-N | 13.0 | 0.07 | 12.5 | 0.21 | * |
| Urea-N:creatinine-N | 9.1 | 0.27 | 9.6 | 0.84 | NS |

* $P< 0.05$; NS, not significant.

\+ Time-period 1, saline, $n$ 2, lysine, $n$ 4; time-period 2, saline, $n$ 4, lysine, $n$ 2.

**Table 3.6.**

*Mean daily urinary N metabolite excretion rates ($mg/kg^{0.75}$ per day) for piglets infused colonically with either physiological saline (9g NaCl/l) or free methionine, while fed with a milk-formula 60% deficient in methionine and 40% deficient in cysteine (S- diet)*

(Mean values with their standard errors for four piglets)

| | Infusion | | | | Statistical Significance |
|---|---|---|---|---|---|
| | Saline | | Methionine | | |
| | Mean | SE | Mean | SE | |
| Time-period 1 | | | | | |
| Total N | 184.9 | 10.42 | 179.8 | 3.72 | NS |
| Urea-N | 152.8 | 10.2 | 141.6 | 5.45 | NS |
| Total N:creatinine-N | 13.9 | 0.96 | 14.8 | 1.80 | NS |
| Urea-N:creatinine-N | 11.5 | 1.14 | 11.7 | 1.56 | NS |
| Time-period 2 | | | | | |
| Total N | 224.6 | 6.35 | 229.6 | 11.84 | NS |
| Urea-N | 172.1 | 4.72 | 192.5 | 13.05 | NS |
| Total N:creatinine-N | 15.1 | 1.40 | 15.9 | 0.80 | NS |
| Urea-N:creatinine-N | 11.6 | 1.39 | 13.2 | 0.52 | NS |

NS, not significant.

For piglets fed the L- diet there were no significant differences ($P>0.05$) in the excretion of urinary N metabolites between those infused with saline and those infused with lysine in either time-period 1 or time-period 2 (Table 3.5). In the piglets infused with lysine the urea-N:creatinine-N ratio in time-period 1 was significantly higher ($P<0.05$), and the total N:creatinine-N ratio in time-period 2 was slightly but significantly lower ($P<0.05$) than in piglets infused with saline.

For piglets fed the S- diet there were no significant differences ($P>0.05$) in the excretion of urinary N metabolites between those infused with saline or those infused with methionine in either time-period 1 or time period 2 (Table 3.6). In time-period 3 the five unoperated piglets were given the S- diet and the excretion of urinary N metabolites by these piglets was significantly ($P<0.05$) greater than that for the eight catheterized piglets which were given the S+ diet (Fig. 3.2).

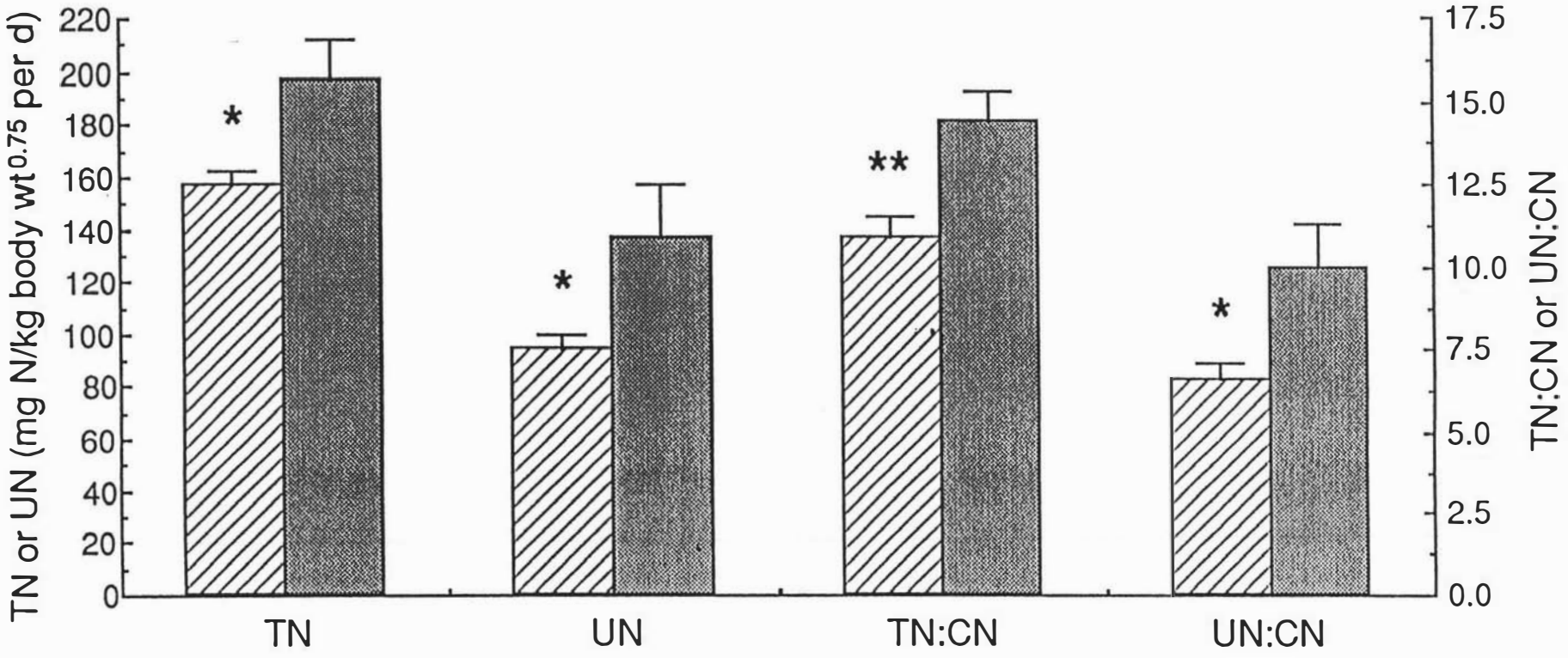


**Figure 3.2.**

Comparison of the mean daily urinary excretion of total N (TN) and urea-N (UN), and the ratios between TN and the mean daily urinary creatinine-N (CN) excretion (TN:CN), and between UN and CN (UN:CN) in time-period 3 for catheterized piglets (hatched; *n* 8) fed with a milk-formula diet balanced for all amino acids except cysteine (S+ diet) and unoperated piglets (shaded; *n* 5) fed with a diet 60% deficient in methionine and 40% deficient in cysteine (S- diet). Values are means with their standard errors represented by vertical bars. Mean values for piglets fed with the S+ diet were significantly different from those for piglets fed with the S- diet: * $P < 0.05$, ** $P < 0.01$.

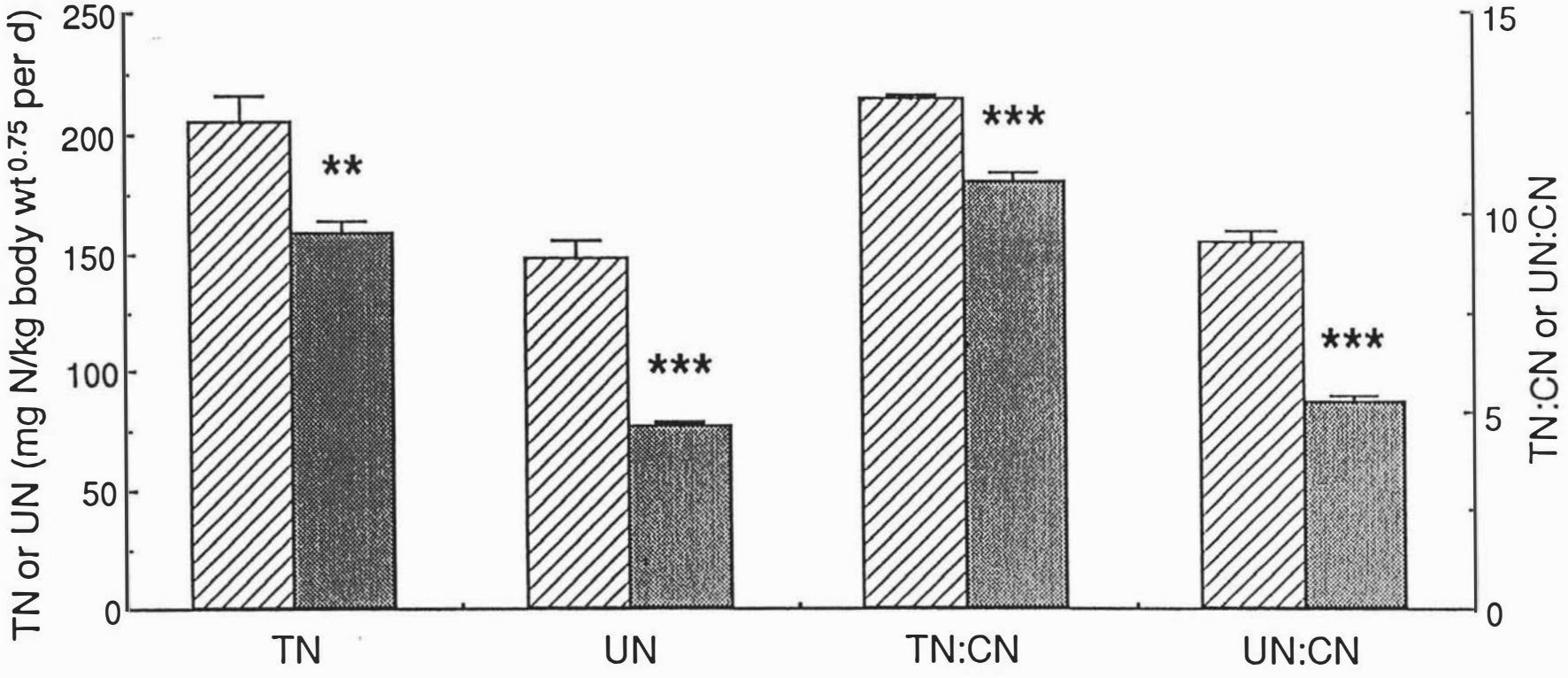


**Figure 3.3.**

Comparison of the mean daily urinary excretion of total N (TN) and urea-N (UN), and the ratios between TN and the mean daily urinary creatinine-N (CN) excretion (TN:CN), and between UN and CN (UN:CN) for catheterized piglets ($n$ 6) fed with a milk-formula diet 40% deficient in lysine (hatched; L- diet) during time-period 2 and the same piglets fed on a milk-formula diet balanced for all amino acids (shaded; L+ diet) during time-period 3. Values are means with their standard errors represented by vertical bars. Mean values for piglets fed with the L- diet were significantly different from those when the same piglets were fed with the L+ diet: ** $P < 0.01$, *** $P < 0.001$.

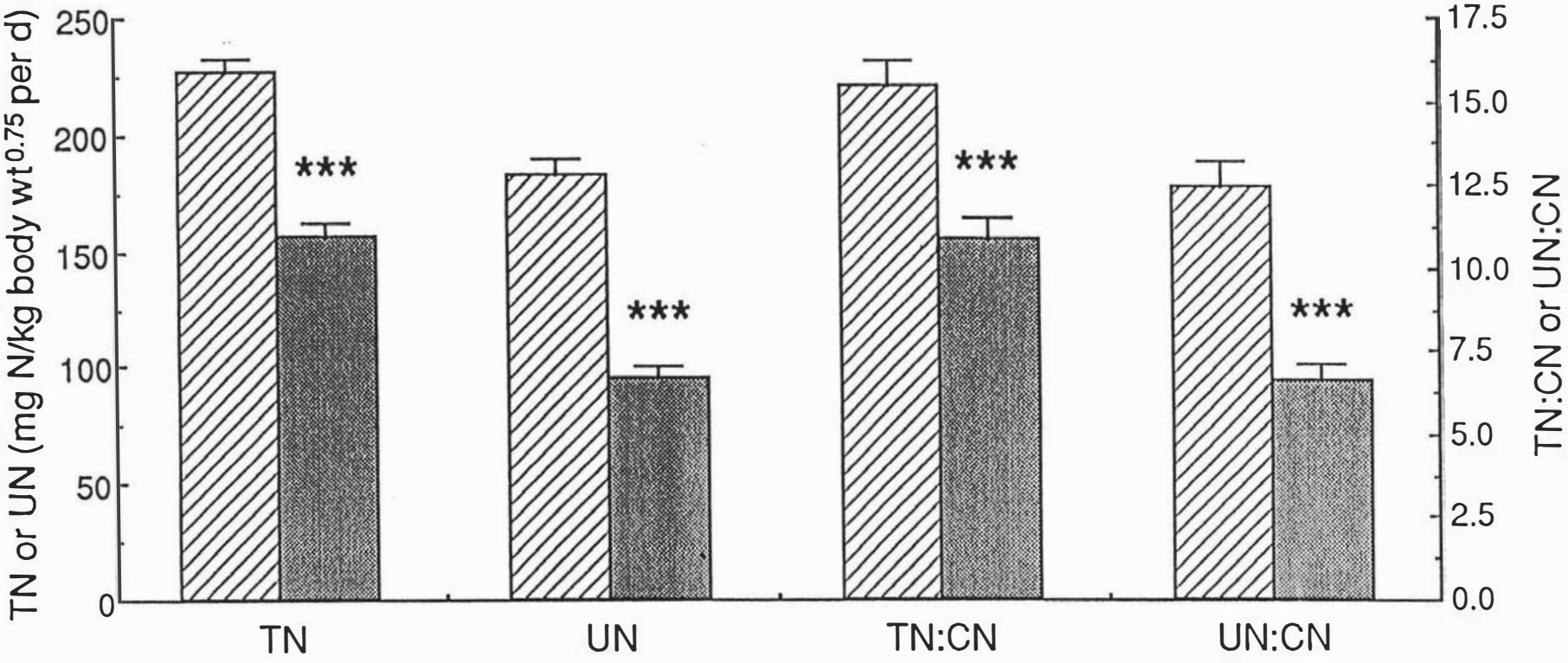


**Figure 3.4.**

Comparison of the mean daily urinary excretion of total N (TN) and urea-N (UN), and the ratios between TN and the mean daily urinary creatinine-N (CN) excretion (TN:CN), and between UN and CN (UN:CN) for catheterized piglets ($n$ 8) fed with a milk-formula diet 60% deficient in methionine and 40% deficient in cysteine (hatched; S- diet) during time-period 2 and the same piglets fed on a milk-formula diet balanced for all amino acids except cysteine (shaded; S+ diet) during time-period 3. Values are means with their standard errors represented by vertical bars. Mean values for piglets fed with the S- diet were significantly different from those when the same piglets were fed with the S+ diet: *** $P < 0.001$.

The excretion of urinary N metabolites by piglets fed on the L- or S- diets in time-period 2 was significantly greater ($P<0.01$) than that from the same pigs fed on the L+ or S+ diets in time-period 3 (Figs. 3.3 and 3.4).

## 3.5 DISCUSSION

The present study was designed to determine whether lysine or methionine can be absorbed in nutritionally significant amounts from the proximal colon of the milk-formula-fed piglet. Piglets given diets deficient in a particular dietary essential amino acid are unable to utilize all the absorbed dietary amino acids for body protein deposition. Excess amino acids are catabolized and the N excreted in the urine. If the deficient amino acids are infused into the proximal large intestine and are absorbed in nutritionally significant amounts, the improvement in dietary amino acid balance and subsequent increase in body protein deposition would be expected to lead to a marked decrease in the urinary excretion of N-containing metabolites.

The piglets were fed on milk-formula diets balanced for all essential amino acids except lysine (L-) or methionine and cysteine (S-), and infused with amounts of amino acid consistent with the expected flow of the amino acid entering the large intestine of piglets given a completely balanced diet. The levels of amino acid infused into the proximal colon represented 13.3% and 12.3% of the lysine and methionine fed/kg liveweight per day respectively. It was anticipated that at this level the infused amino acids, if absorbed,would have been sufficient to produce a response in the urinary N metabolite excretion. An increase in the amount of deficient amino acid in the piglet's metabolic amino acid pool should have enabled the other amino acids to be utilized more efficiently, thus providing a sensitive assay. Surgical intervention and the presence of catheters in the caecum and proximal colon appeared to have no effect on urinary N metabolite excretion (Fig 3.1).

It is important to note that the site for potential amino acid absorption was not clearly defined in the present study. Although the catheter was introduced into the caecum and the tip of the catheter placed well past the ileo-caecal junction, a possible back flow of amino acids into the terminal ileum could have occurred and resulted in the absorption of infused amino acids from the distal small intestine. The absence of HA in ileal contents following infusion of this amino acid into the large intestine indicated that back flow did not occur to any significant extent. Absorptive capacity may vary throughout the large intestine. Olszewski and Buraczewski (1978) observed

amino acid absorption in the caecum of growing pigs, while in other studies (James and Smith, 1976) evidence was found of amino acid uptake by mucosa from the proximal large intestine. It is possible that the infusate used in the present study did reflux into the caecum, though the main region for potential absorption would probably have been the proximal colon.

The use of ostomy plates and bags in the present study ensured that coprophagy did not occur, which would have led to difficulties in the interpretation of the metabolite excretion data. In addition, the use of ostomy bags ensured the collection of uncontaminated urine. While total collection of urine could not be assumed in the present study, with the possible loss of urinary nitrogen as ammonia before the urine was acidified, care was taken to minimise any urinary nitrogen loss. The overall mean for the individual piglet's coefficient of variation for daily urinary creatinine-N excretion was 14.7%. An average coefficient of variation of 7% for creatinine excretion was determined under conditions of complete urine collection from rats (Das and Waterlow, 1974). Since the coefficient of variance observed in the present study was higher than would be expected based on random variation in creatinine excretion, urine collection may not have been complete. For the purposes of relative comparison, however, the urinary nitrogen metabolite excretion values and their ratios to creatinine determined in the present study were considered adequate.

The validity of this experiment also relied on the assumption that the milk-formula diets were sufficiently deficient to allow for a significant response in urinary N metabolite excretion consequent upon possible absorption of the limiting amino acid. Given, from a comparison in time-period 2, that there appeared to be no significant effect of surgical intervention on the excretion of urinary N metabolites (Fig 3.1), in time-period 3 a comparison was made between the mean daily urinary N metabolite excretion rates of the catheterized piglets given the S+ diet, and the unoperated piglets given the S- diet (Fig. 3.2). The mean daily urinary N metabolite excretion rates for the piglets given the S+ diet were significantly lower than those for piglets given the S- diet which indicates that the S- diet was deficient in sulphur amino acids. A similar direct comparison with the L- and L+ diets was not made.

Further evidence that the L- and S- diets were suitably deficient is shown by comparison of the mean daily urinary N metabolite excretion rates for piglets given either the L- or S- diets during time-period 2 and the excretion rates for the same piglets given either the L+ or S+ diets during time-period 3 (Figs. 3.3 and 3.4). In time-

periods 1 and 2 when the piglets were given the L- and S- diets, urinary N metabolite excretion showed a significant increase over time. However, in time-period 3 there was a significant reduction in mean daily urinary N metabolite excretion when the piglets received balanced diets compared with those in time-period 2 when they received deficient diets. Thus, the apparent increase in urinary N metabolite excretion over time was reversed and excretions were significantly reduced when balanced diets were fed to the piglets. It is concluded that during time-periods 1 and 2 the piglets were in a state of amino acid deficiency so that there would have been a significant decrease in urinary N metabolite excretion had infused amino acids been absorbed from the proximal colon.

The mean daily urinary N excretion rates for piglets given either diets L- and S- and infused with physiological saline or the deficient amino acids were similar, indicating that lysine and methionine were not absorbed in significant amounts from the proximal colon of the milk-formula-fed piglet.

Previous studies have shown that amino acids are not absorbed from the hindgut of growing pigs (Zebrowska, 1973, 1975, 1978; Just *et al.* 1981; Schmitz *et al.* 1991). In contrast to these studies, Olszewski and Buraczewski (1978) and Niiyama *et al.* (1979) suggested that amino acids may be absorbed from the hind-gut of growing pigs. Olszewski and Buraczewski (1978) measured the disappearance of infused amino acids from an isolated pig caecum *in situ*. Microbiological degradation was limited by flushing the caecum with antibiotics. They concluded that as amino acids disappeared selectively from the caecal sac, an active transport system must be present rather than absorption by simple diffusion. This is contradicted, however, by other studies and reviews stating that no active transport systems exist in the hindgut epithelium (Batt and Schachter, 1969; Binder, 1970; Munck, 1981).

Niiyama *et al.* (1979) measured the absorption of microbial amino acids from the colon of growing pigs by determining the $^{15}N$ concentration of free amino acids in colonic venous blood after infusion of $^{15}N$-labelled micro-organisms into the caecum. Although $^{15}N$-amino acids were located in the blood, $^{15}N$-ammonia may have been absorbed and subsequently converted to amino acids within the body. Niiyama *et al.* (1979) concluded, however, that because of the difficulty in transferring N from ammonia to an amino acid group, $^{15}N$-amino acids found in the venous blood supply of the colon were directly derived from the infused micro-organisms.

Olszewski and Buraczewski (1978) and Niiyama *et al.* (1979) have shown that

amino acids may be absorbed into the mucosa, serosa and possibly the blood supply of the caecal-colon region. They did not, however, measure any metabolic variables that would identify whether the amounts of amino acids absorbed were of nutritional significance.

While it appears that amino acids are unlikely to be absorbed in the hind-gut of growing animals, there is some evidence that amino acids may be absorbed from either the caecal or colonic epithelium in several species of young animal (Batt and Schachter, 1969; James and Smith, 1976; Heine *et al.* 1987).

*In vitro* studies using isolated segments of new-born and neonatal piglet proximal colon detected the movement of methionine into the epithelium (James and Smith, 1976; Smith and James, 1976; Jarvis *et al.* 1977). Sepulveda and Smith (1979) identified two mechanisms for entry of neutral amino acids into the new-born piglet proximal colon using *in vitro* methodology. Although these studies demonstrated a definite movement of amino acids into the hind-gut epithelial mucosa, there was no evidence to suggest that the amounts absorbed were nutritionally significant. In addition, all the studies indicated diminishing absorptive capacity with age. James and Smith (1976) noted that absorption was reduced considerably by 4 days and was almost undetectable by 10 days of age. This loss of activity has been attributed to a change in the epithelial cell structure of the hindgut soon after birth (Holdsworth and Hastings-Wilson, 1967; Henin and Smith, 1976; James and Smith, 1976; Potter and Lester, 1984).

The uptake of amino acids into the colonic mucosa and serosa was not directly determined in the present work. However, it can be concluded that free lysine and methionine are not absorbed in nutritionally significant amounts from the proximal colon of the milk-formula-fed piglet (15-31 day old). Further research is needed to evaluate whether this is true for all amino acids, and to determine whether the caecal epithelium alone is capable of absorbing amino acids. The results of this, and other studies, support the use of an ileal digestibility assay with young milk-formula-fed piglets.

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# CHAPTER 4

# CORRECTION FOR AMINO ACID LOSS DURING ACID HYDROLYSIS.

## I. LOSSES IN A PURIFIED PROTEIN

*Submitted - Analytical Biochemistry*

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Fundamental to the determination of amino acid requirements for infants based on the amino acid composition of human milk, is the knowledge that the amino acid composition of human milk has been accurately determined. The routine 24 hour hydrolysis of protein in acid prior to amino acid detection may lead to an underestimation of amino acid content due to degradation of amino acids. The following study describes a method for correcting for losses of amino acids during acid hydrolysis.

## 4.1 ABSTRACT

Hydrolysing a protein in acid for a single hydrolysis interval, normally 24 hours, will lead to inaccurate estimates of the amino acid composition of that protein due to an effect of the time of hydrolysis on peptide bond cleavage and amino acid degradation. The simultaneous yield and decay of amino acids during the hydrolysis of a protein can be described by a compartmental model with parameters for the hydrolysis and loss rates specific to each amino acid in a protein. The amino acid composition of the protein prior to hydrolysis can be determined by non-linear regression of data derived from multiple hydrolysis intervals. In the present study egg white lysozyme was hydrolysed in 6 M HCl for 18 different hydrolysis intervals (range 2-141 hours) using the conventional duplicate hydrolyses/interval system. Hydrolysis and loss rates were determined for each amino acid. Increasing the number of hydrolysis intervals prior to the maximum point on the hydrolysis curve, and including an hydrolysis interval greater than 100 hours increased the accuracy with which the hydrolysis and loss rates were estimated. Most of the amino acids underwent some degree of loss during hydrolysis. Of particular note was the loss rate for cysteic acid, which was greater than that found for serine which is commonly regarded as an acid-labile amino acid. The determined amino acid composition of the protein, based on the non-linear regression of the data from 4 different series of hydrolysis intervals was compared with the known amino acid composition (sequencing). Using the routine duplicate sampling system, a non-linear regression including 10 hydrolysis intervals (2, 6, 10, 14, 18, 22, 26, 30, 60, 141 hours) resulted in a mean amino acid recovery of 100% (range, 94-110%) and provided an acceptable compromise between accuracy and the cost of analysis.

## 4.2 INTRODUCTION

Prior to determining the amino acid content of a protein, the amino acids must be released, usually by hydrolysing the protein in hot acid for 24 hours (Finley, 1985). After hydrolysis, the free amino acids can be detected. Variations in the ease of peptide bond cleavage and differences in the acid-stability of the amino acids have a significant effect on estimates of the amino acid composition of a protein. For example, peptide bonds involving valine, isoleucine and leucine are difficult to hydrolyse (Blackburn, 1978), and obtaining a maximum yield for these amino acids may require hydrolysis times considerably longer than 24 hours for some proteins. In contrast, acid-labile amino acids such as serine and threonine can be partially destroyed before measurement is made. In both these situations the yield of amino acids determined in a protein hydrolysate after 24 hours hydrolysis may be inaccurate.

Although not routinely applied, correction for losses of amino acids during hydrolysis or for the further release of amino acids from resistant peptide bonds is possible. A method for determining the amount of valine, isoleucine and leucine in a protein is to adopt multiple hydrolysis intervals and extrapolate the amino acid composition data to an infinite time (Blackburn 1978). Likewise, compensation for amino acid degradation may be made by calculation to zero time assuming first order kinetics (Hirs et al., 1954; Slump, 1969). By extrapolation to time zero or using the maximum point on an hydrolysis time curve, correction factors can be derived for the adjustment of subsequent 24 hour hydrolyses (Kohler and Palter, 1967; Slump, 1980; Gehrke et al., 1985; Rowan et al., 1992).

The processes of hydrolysis and degradation of amino acids occur simultaneously, however, and a given point on a curve derived from the measurement of amino acids at different hydrolysis intervals, is not a true measure of amino acid yield. Where there are significant amino acid losses, extrapolations taken directly from points on an hydrolysis curve are biased.

Robel and Crane (1972) proposed an alternative method for determining the true amino acid composition of a protein, using a non-linear least squares extrapolation to time zero, of the amino acid composition data from a series of hydrolysis intervals. Their model follows the theory of compartmental analysis (Matis and Hartley, 1971) with three distinct compartments (states). State A represents the amount of an amino acid in a protein-bound form. As amino acids are cleaved from the protein they move to State B where detection is possible. Susceptible amino acids that are converted to

unidentifiable compounds or simply degraded during acid hydrolysis shift to State C and are no longer identifiable. The amount of amino acid detectable in state B at time t is therefore determined by the amount of amino acid present prior to hydrolysis (t=0), the release of that amino acid during hydrolysis (from State A to B) and any loss of the amino acid (from State B to C). This relationship can be described by the following equation:

$$B(t) = \frac{Ao\ h}{h - l}(e^{-lt} - e^{-ht}) + \epsilon$$

where,

| | | |
|---|---|---|
| B(t) | = | amino acids measured at time t. |
| *Ao* | = | the original level of amino acids in the protein prior to hydrolysis, ie. at t = 0. |
| *h* | = | the rate at which the protein bound amino acids are hydrolysed to a free, and measurable form. |
| *l* | = | the rate at which amino acids are degraded to an undetectable form. |
| ε | = | the random residual error that accounts for measurement error among other factors. |

The model assumes that the rate of destruction of labile amino acids while still in a peptide bond is minor (Robel and Crane, 1972). It is also assumed that the hydrolysis rate (*h*), expressed as a proportion of the protein-bound amino acid per hour, and the loss rate (*l*), expressed as a proportion of the detectable amino acids per hour, are constant fractions of the amounts of amino acid in State A and State B, respectively. Estimates of *h*, *l*, and *Ao* for a particular protein can be derived using non-linear least-squares regression of a series of B(t) values measured at different hydrolysis times. The model is applicable to all amino acids including those that may not exhibit any loss rate.

Robel and Crane (1972) used this model to estimate the amino acid composition of egg-white lysozyme. Estimates of *h*, *l* and *Ao* were derived and when compared to the amino acid composition of lysozyme determined independently by sequencing (Canfield, 1965), the *Ao* values ranged from 90-110% of the true (sequence) values with

an average recovery of 99.1%. Robel and Crane (1972) used 5 hydrolysis times with the mean of 5 replicate hydrolyses per time to create an hydrolysis curve for each amino acid.

The cost of accurately determining the amino acid composition of a protein, using the Robel and Crane (1972) method, is a function of the number of replicates multiplied by the number of hydrolysis intervals. Although more replicates per hydrolysis time will increase the precision of measurement, more hydrolysis intervals should improve the accuracy with which parameters of the compartment model are estimated. In practice a compromise needs to be reached between accuracy and cost.

An objective of the present study was to ascertain, using the routinely adopted duplicate hydrolysis system, how accurately the amino acid composition of a purified protein could be determined based on 5 hydrolysis intervals and to what extent the accuracy could be improved as the number of hydrolysis intervals was increased. The extent to which accuracy is affected by varying the range of hydrolysis intervals was also examined. In addition, estimates of *Ao*, *h* and *l* for the sulphur amino acids, which were not presented by Robel and Crane (1972), were determined following performic oxidation and acid hydrolysis.

## 4.3 EXPERIMENTAL

### 4.3.1 **Chemical Analysis**

Samples (2-4 mg) of egg-white lysozyme (Sigma, St Louis, MO, USA Code: L-6876) were accurately weighed into 36 x 10 ml acid-washed pyrolysed (650°C, overnight) glass test tubes each containing 1 ml of glass-distilled 6 M HCl (containing 1% phenol w/v). An internal standard (50 µl of 40 mM norleucine; Sigma, St Louis, MO, USA Code: N - 8513) was also added to allow for adjustment due to errors in quantitation of the amino acids. The stability of norleucine during acid hydrolysis was confirmed in a preliminary study (A.J. Darragh, unpublished). The tubes were sealed under vacuum and duplicates hydrolysed at 110 ± 2°C for either 2, 4, 6, 8 ,10, 12, 14, 16, 18, 20, 22, 24, 26, 30, 50, 60, 72 and 141 hours. After hydrolysis the acid was removed under vacuum (Automatic Speedvac Concentrator AS290, Savant Instruments Inc, Farmingdale, NY) and the amino acids dissolved in loading buffer (1 ml of 0.2 M sodium citrate, pH 2.2). Samples that had been hydrolysed for less than 6 hours were dissolved in the loading buffer and deproteinised by ultrafiltration using a micropartition system (10,000 MW cutoff, Amicon Division, Grace & Co, Danvers, MA,

USA). The samples were loaded onto a Pharmacia LKB - Alpha Plus Amino Acid Analyser (Cambridge, England). The amino acids were separated by ion-exchange chromatography and detected following reaction with ninhydrin.

The lysozyme was also analysed for cysteine and methionine. Two ml of freshly prepared performic acid (1 part 30% $H_2O_2$ to 9 parts 88% formic acid) was added to 36 acid-washed, pyrolysed test tubes each containing 2-4 mg samples of lysozyme. The tubes were kept at 0°C for 16 hours, after which time 300 µl of 48 % HBr was added to each tube. The samples were dried under vacuum and 50 µl of 40 mM norleucine added to each tube. The samples were hydrolysed under vacuum in 1 ml of 6 M HCl with the same conditions and using the same time intervals as described above. After hydrolysis and acid removal, 2 ml of loading buffer was added to each tube. Samples hydrolysed for less than 6 hours were deproteinised as described above. The samples were loaded on to an Ion-exchange HPLC System (Millipore Corporation, Waters Chromatography Division, Milford, MA, USA), utilising post-column derivitisation with *O*-phthalaldehyde and fluorescence detection. Cysteine and methionine were detected as cysteic acid and methionine sulphone, respectively. Tryptophan, which is destroyed during acid hydrolysis, was not determined.

### 4.2.3 **Data Analysis**

The duplicate analyses for each time interval were averaged to provide B(t) values. Four sets of B(t) values pertaining to four different series of hydrolysis intervals were chosen for computation. **I:** the intervals suggested by Robel and Crane (1972) (4, 24, 50, 72 and 141 hours); **II:** a series of 10 hydrolysis intervals (2, 6, 10, 14, 18, 22, 26, 30, 50 and 60 hours) ; **III:** a second series of 10 hydrolysis intervals which included a 141 hour hydrolysis interval (2, 6, 10, 14, 18, 22, 26, 30, 60 and 141 hours), and **IV:** the entire sequence of hydrolysis intervals.

The best fit equation as defined by the parameters $h$, $l$ and $Ao$ was computed for each amino acid using a non-linear least squares regression procedure constrained to a defined parameter space ($Ao>0$, $h>0$, $l>=0$). The Marquardt method was adopted for analysis. The non-linear regression procedure (SAS, 1985) and the partial derivatives of the non-linear equation required by the Marquardt method are given in Appendix 4a (page 140).

The estimates of the three parameters are assumed to be asymptotically normal. The asymptotic standard error (ASE) of each estimate of the parameter values is a

function of the unknown residual variance. The SAS procedure calculates an estimate of ASE based on the Mean Square Residual from the non-linear least squares. The estimate of the mean square residual will vary according to which sequence of hydrolysis intervals is chosen, although the true standard error is a parameter with a fixed value. Accordingly, and to allow a valid comparison of the accuracy with which the parameters *h*, *l*, and *Ao* were estimated using the different series of hydrolysis intervals, all ASE values shown in this paper are presented as scalar multiples of the unknown standard deviation.

## 4.4 RESULTS

The overall mean difference between duplicates within samples (expressed as a proportion of the mean) determined over all amino acids and hydrolysis times, was 5.4%. The curves generated using the multiple hydrolysis times are presented in Figures 4.1 and 4.2. For most of the amino acids, the maximum point on the hydrolysis time curve occurred before 10 hours, with the exception of valine, isoleucine, and cysteic acid where the maxima occurred between 15 and 20 hours.

The estimates of *h* and *l* determined after analysis of all four series of hydrolysis intervals are given in Tables 4.1 and 4.2, respectively. In general, as the number of hydrolysis times increased, the estimates of *h* and *l* decreased and increased, respectively. In addition, the ASE's of the estimates decreased.

In practice, iterative techniques such as non-linear least squares algorithms can sometimes diverge, failing to give realistic solutions. For example, the series I estimates of *h* for leucine, tyrosine, phenylalanine, and lysine are unrealistically large. Inspection of the partial derivatives in Appendix 4a, shows that large values of *h* will give partial derivatives that approach zero. This may preclude further change in the estimate of *h*. Sometimes, the partial derivative approaches so close to zero that the Jacobian matrix can become singular. The non-linear least squares procedure will continue without further estimation of *h* and the SAS output will not provide an ASE for these *h* estimates. This occurred for tyrosine when series I was used. Estimates of the parameters *l* and *Ao* associated with these high *h* values should be interpreted with caution. Likewise, the accuracy of *h* estimates having large ASE values (series II) may be in doubt.

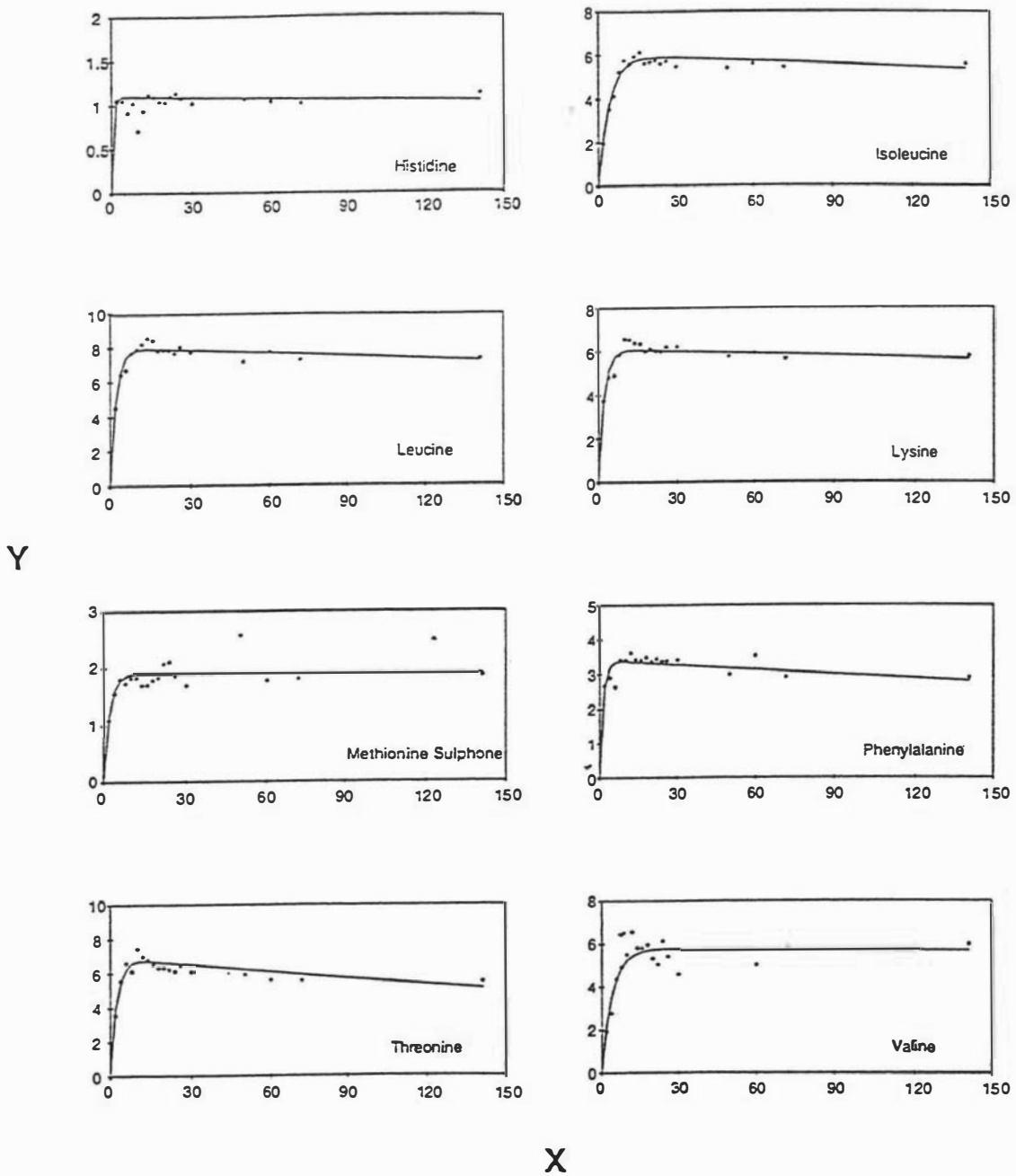


**Figure 4.1.**

Effect of hydrolysis time (X axis, hours) during amino acid analysis on the mean yield of amino acids (Y axis, moles of amino acid per mole of protein) in egg white lysozyme. The mean values for duplicate samples (♦) and the line of best fit determined using estimates of $h$, $l$ and $Ao$ pertaining to the data are plotted.

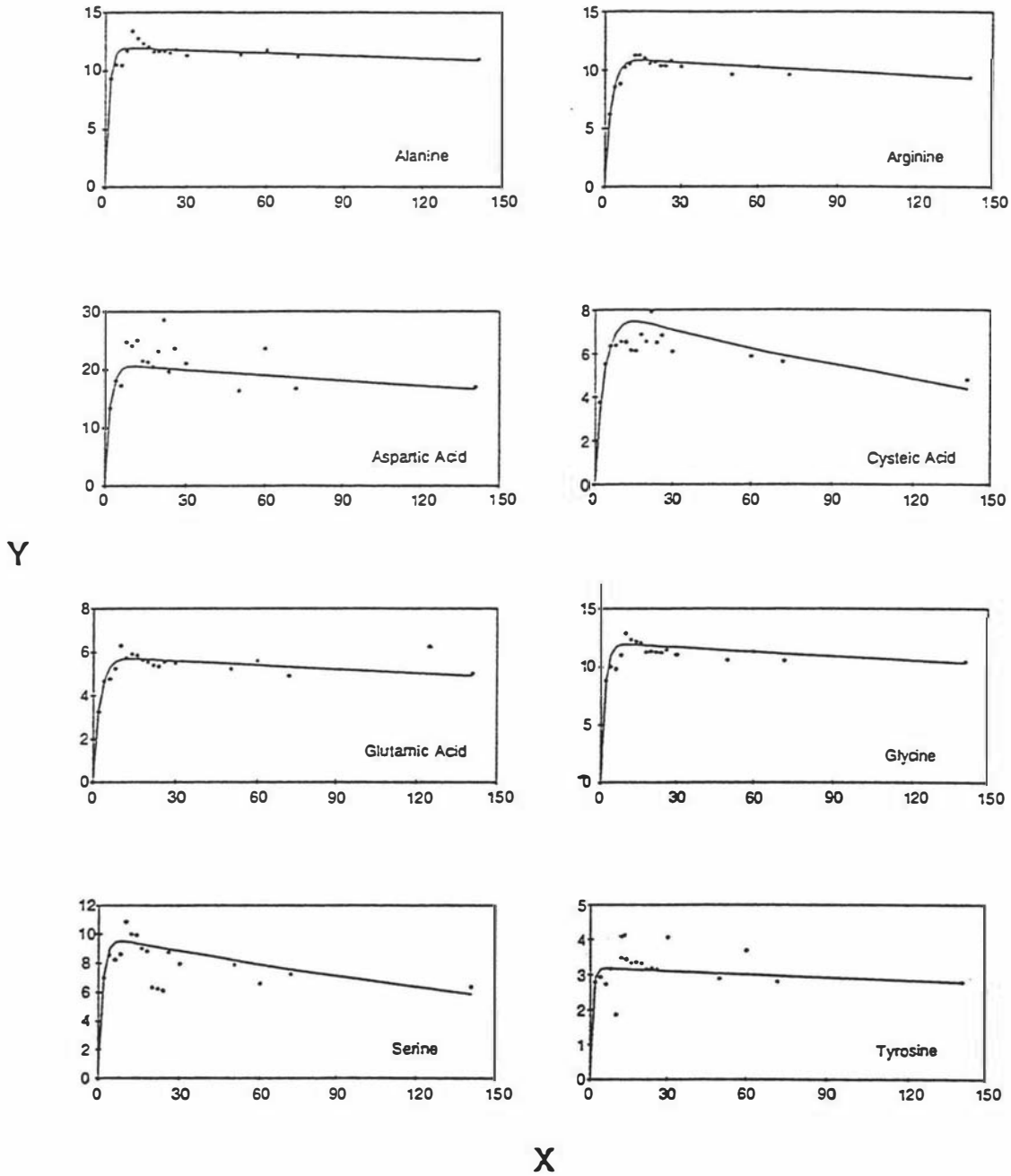


**Figure 4.2.**

Effect of hydrolysis time (X axis, hours) during amino acid analysis on the mean yield of amino acids (Y axis, moles of amino acid per mole of protein) in egg white lysozyme. The mean values for duplicate samples (♦) and the line of best fit determined using estimates of *h*, *l* and *Ao* pertaining to the data are plotted.

**Table 4.1.**

*Hydrolysis (h) rates (fraction of A* hydrolysed per hour) ± ASE' for amino acids in egg-white lysozyme during acid hydrolysis, and determined by non-linear least squares regression of different sequences of hydrolysis intervals (I-IV).*

| | I# | | II§ | | III¶ | | IV° | |
|---|---|---|---|---|---|---|---|---|
| **Amino Acid** | *h* | ASE | *h* | ASE | *h* | ASE | *h* | ASE |
| Glutamic Acid | 0.853 | 2.0003 | 0.429 | 0.2201 | 0.391 | 0.1795 | 0.423 | 0.1579 |
| Glycine | 0.628 | 0.3554 | 1.965 | 4.0377 | 0.733 | 0.1959 | 0.634 | 0.1398 |
| Serine | 1.055 | 2.6740 | 0.636 | 0.2289 | 0.678 | 0.2143 | 0.652 | 0.1801 |
| Threonine | 0.620 | 0.6266 | 0.375 | 0.1668 | 0.389 | 0.1676 | 0.418 | 0.1368 |
| Aspartic Acid | 1.027 | 1.0850 | 0.587 | 0.1037 | 0.606 | 0.0912 | 0.545 | 0.0128 |
| Alanine | 0.958 | 1.3793 | 0.872 | 0.3058 | 0.837 | 0.2395 | 0.785 | 0.3224 |
| Valine | 0.171 | 0.0992 | 0.203 | 0.0966 | 0.219 | 0.0936 | 0.233 | 0.2732 |
| Isoleucine | 0.264 | 0.1493 | 0.188 | 0.0880 | 0.200 | 0.0824 | 0.232 | 0.1199 |
| Leucine | 17.365 | 0.9136 | 0.360 | 0.0305 | 0.383 | 0.1258 | 0.434 | 0.0455 |
| Tyrosine | 38.418 | -" | 2.072 | 22.2922 | 1.402 | 2.8974 | 1.233 | 0.1742 |
| Phenylalanine | 31.816 | 22.0503 | 1.971 | 17.8746 | 0.744 | 0.6889 | 0.736 | 1.9462 |
| Histidine | 6.661 | 13.6524 | 1.970 | 45.9393 | 1.972 | 2.4528 | 1.528 | 0.2326 |
| Lysine | 17.350 | 1.1506 | 1.774 | 6.2122 | 0.435 | 0.1970 | 0.461 | 0.8778 |
| Arginine | 0.470 | 0.1979 | 0.403 | 0.1103 | 0.388 | 0.0960 | 0.408 | 0.0869 |
| Methionine** | 0.273 | 0.3817 | 1.719 | 15.0988 | 0.474 | 0.7378 | 0.462 | 0.5804 |
| Cysteine++ | 0.277 | 0.1178 | 0.282 | 0.1250 | 0.349 | 0.1500 | 0.275 | 0.1061 |

* Amino acids in protein-bound form during acid hydrolysis.

' Scalar multiple of the unknown standard deviation.

# 4, 24, 50, 72 and 141 hours.

§ 2, 6, 10, 14, 18, 22, 26, 30, 50 and 60 hours.

¶ 2, 6, 10, 14, 18, 22, 26, 30, 60 and 141 hours.

° 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30, 50, 60, 72 and 141 hours.

" It was not possible to calculate the ASE for this estimate as the Jacobian Matrix was singular (SAS, 1985).

** Detected as methionine sulphone.

++ Detected as cysteic acid.

**Table 4.2.**

*Loss (l) rates (fraction of B* hydrolysed per hour) ± ASE' for amino acids in egg-white lysozyme during acid hydrolysis, and determined by non-linear least squares regression of different sequences of hydrolysis intervals (I-IV).*

| | I# | | II§ | | III¶ | | IV° | |
|---|---|---|---|---|---|---|---|---|
| **Amino Acid** | *l* | ASE | *l* | ASE | *l* | ASE | *l* | ASE |
| Glutamic Acid | 0.0001 | 0.00228 | 0 | 0.00440 | 0.0008 | 0.00165 | 0.0012 | 0.00152 |
| Glycine | 0.0009 | 0.01083 | 0 | 0.00309 | 0.0006 | 0.00076 | 0.0011 | 0.00071 |
| Serine | 0.0028 | 0.00166 | 0.0048 | 0.00547 | 0.0031 | 0.00130 | 0.0037 | 0.00115 |
| Threonine | 0.0013 | 0.00205 | 0.0027 | 0.00419 | 0.0017 | 0.00157 | 0.0022 | 0.00143 |
| Aspartic Acid | 0.0014 | 0.00068 | 0 | 0.00263 | 0.0006 | 0.00048 | 0.0017 | 0.00046 |
| Alanine | 0.0002 | 0.00104 | 0 | 0.00402 | 0.0005 | 0.00076 | 0.0007 | 0.00069 |
| Valine | 0 | 0.00211 | 0.0023 | 0.00569 | 0 | 0.00166 | 0.0001 | 0.00150 |
| Isoleucine | 0.0003 | 0.00210 | 0.0017 | 0.00570 | 0.0006 | 0.00176 | 0.0008 | 0.00161 |
| Leucine | 0 | 0.00211 | 0.0020 | 0.00089 | 0.0007 | 0.00119 | 0.0008 | 0.00112 |
| Tyrosine | 0.0005 | 0.03340 | 0 | 0.02486 | 0.0005 | 0.00294 | 0.0010 | 0.00268 |
| Phenylalanine | 0.0001 | 0.00324 | 0 | 0.02444 | 0.0011 | 0.00283 | 0.0014 | 0.00268 |
| Histidine | 0 | 0.00884 | 0 | 0.03610 | 0 | 0.01299 | 0.0005 | 0.00694 |
| Lysine | 0 | 0.00281 | 0 | 0.01335 | 0.0005 | 0.00156 | 0.0006 | 0.00148 |
| Arginine | 0.0011 | 0.00121 | 0 | 0.00244 | 0.0008 | 0.00091 | 0.0012 | 0.00085 |
| Methionine- | 0.0018 | 0.00589 | 0 | 0.01881 | 0.00001 | 0.004883 | 0.00001 | 0.005303 |
| Cysteine** | 0.0045 | 0.00216 | 0.0055 | 0.00484 | 0.0045 | 0.00019 | 0.0044 | 0.00215 |

* Amino acids in free form during acid hydrolysis.

' Scalar multiple of the unknown standard deviation.

# 4, 24, 50, 72 and 141 hours.

§ 2, 6, 10, 14, 18, 22, 26, 30, 50 and 60 hours.

¶ 2, 6, 10, 14, 18, 22, 26, 30, 60 and 141 hours.

° 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30, 50, 60, 72 and 141 hours.

- Detected as methionine sulphone.

** Detected as cysteic acid.

**Table 4.3.**

*Estimates of the amino acid composition[*] of egg-white lysozyme (± ASE[•]) determined by non-linear least squares regression of different sequences of hydrolysis intervals (I-IV) compared with the conventional 24 hour hydrolysis value and the composition based on sequencing[#]*

| | I[§] | | II[¶] | | III[✪] | | IV[-] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Amino Acid** | *Ao* | ASE | *Ao* | ASE | *Ao* | ASE | *Ao* | ASE | 24 Hr[••] | **Actual** |
| Glutamic Acid | 5.1 | 0.96 | 5.6 | 0.68 | 5.8 | 0.51 | 5.8 | 0.39 | 5.3 | 5 |
| Glycine | 11.6 | 0.99 | 7.2 | 0.61 | 11.8 | 0.46 | 12.1 | 0.37 | 10.4 | 12 |
| Serine | 9.2 | 1.07 | 10.0 | 1.02 | 9.8 | 0.50 | 9.8 | 0.39 | 8.4 | 10 |
| Threonine | 6.4 | 1.01 | 6.9 | 0.76 | 6.8 | 0.53 | 6.9 | 0.41 | 6.3 | 7 |
| Aspartic Acid | 19.5 | 1.01 | 19.9 | 1.00 | 19.7 | 0.51 | 21.0 | 0.41 | 20.5 | 21 |
| Alanine | 11.3 | 0.96 | 11.8 | 0.92 | 12.0 | 0.46 | 12.0 | 0.37 | 11.3 | 12 |
| Valine | 5.7 | 0.99 | 6.0 | 0.98 | 5.8 | 0.61 | 5.8 | 0.48 | 5.8 | 6 |
| Isoleucine | 5.7 | 0.95 | 6.1 | 1.06 | 6.0 | 0.67 | 6.0 | 0.51 | 5.4 | 6 |
| Leucine | 5.4 | 0.96 | 8.3 | 0.87 | 8.2 | 0.55 | 8.0 | 0.45 | 7.5 | 8 |
| Tyrosine | 3.0 | 0.72 | 1.9 | 0.97 | 3.1 | 0.49 | 3.2 | 0.38 | 2.8 | 3 |
| Phenylalanine | 2.9 | 0.31 | 1.9 | 0.97 | 3.5 | 0.51 | 3.4 | 0.41 | 3.2 | 3 |
| Histidine | 0.9 | 0.70 | 0.7 | 0.74 | 1.1 | 0.53 | 1.1 | 0.37 | 1.1 | 1 |
| Lysine | 4.1 | 0.97 | 3.7 | 0.98 | 6.2 | 0.60 | 6.1 | 0.50 | 5.6 | 6 |
| Arginine | 10.7 | 0.99 | 10.8 | 0.76 | 10.9 | 0.55 | 11.0 | 0.46 | 10.3 | 11 |
| Methionine[++] | 2.3 | 1.02 | 1.2 | 0.65 | 1.9 | 0.56 | 1.9 | 0.42 | 1.9 | 2 |
| Cysteic Acid[##] | 8.3 | 1.27 | 8.0 | 1.13 | 7.8 | 0.78 | 8.0 | 0.77 | 6.5 | 8 |

[*] *Ao* expressed as moles of the amino acid per mole of lysozyme protein.

[•] Scalar multiple of the unknown standard deviation.

[#] Canfield (1965).

[§] 4, 24, 50, 72 and 141 hours.

[¶] 2, 6, 10, 14, 18, 22, 26, 30, 50 and 60 hours.

[✪] 2, 6, 10, 14, 18, 22, 26, 30, 60 and 141 hours.

[-] 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30, 50, 60, 72 and 141 hours.

[••] Mean value of duplicate analyses.

[++] Detected as methionine sulphone.

[##] Detected as cysteic acid

The amino acid composition of the protein, represented by the *Ao* estimates derived from statistical analysis of the four series of hydrolysis intervals, is compared with compositional data based on sequencing (Canfield, 1965) in Table 4.3. The amino acid composition of lysozyme based on the mean value for duplicate 24 hour hydrolyses (conventional approach) is also given in Table 4.3 for comparison with the non-linear derived estimates of *Ao*.

Due to inadequate chromatographic resolution, estimates of *Ao* for proline were not obtained. As a result of this, the estimate of *Ao* for glutamic acid, which elutes immediately before proline, may have been overestimated. A recovery of 116% for glutamic acid (in series IV) when compared to the protein sequence data supports this. The chromatograph peak representing phenylalanine was also poorly resolved which may have resulted in overestimation of the amount of this amino acid for series III and IV. Analysis of series I and II, however, provided an estimate of the amount of phenylalanine that compared favourably with the protein sequence data.

When compared with the protein sequence data (with the exception of glutamic acid and phenylalanine), series I, II, III and IV gave average recoveries of 93% (range: 68-115%), 86% (range: 60-104%), 100% (range: 94-110%) and 100% (range: 95-110%), respectively. The ASE's for the estimates of *Ao* decreased considerably as the number of hydrolysis intervals increased from 5 to 18. The 24 hour hydrolysis values (excluding glutamic acid and phenylalanine) gave an average recovery of 93% (range, 81-110%).

## 4.5 DISCUSSION

In the present study, estimates of the parameters *h*, *l* and *Ao* were computed for each amino acid in egg-white lysozyme by the non-linear least squares regression of data based on several hydrolysis intervals. Previously, Robel and Crane (1972) have shown satisfactory recoveries of amino acids (when compared with protein sequence data) when five hydrolysis intervals were analysed using this procedure. When the same five hydrolysis intervals were used in the present study, however, amino acid recoveries were lower than those reported by Robel and Crane (1972). In contrast to the analysis of duplicate samples at each hydrolysis interval in the present study, Robel and Crane (1972) used five replicates per hydrolysis interval. This would have improved the precision of their mean amino acid concentration at each hydrolysis interval resulting in more accurate amino acid recoveries.

Just as the precision of the average measurement for each hydrolysis interval

may be limited by a low number of replicates, the accuracy with which the model parameters are estimated may be limited by the number of hydrolysis intervals used. Adopting a duplicate sampling system and a small number (5) of hydrolysis intervals resulted in unrealistic estimates for the least squares procedure. This was evident in the large estimates of $h$ for some amino acids, in particular for tyrosine where the Jacobian matrix became singular during statistical analysis of series I.

With the duplicate sampling procedure more accurate estimates of $h$ and $l$ were obtained by increasing the number of hydrolysis intervals. The accuracy with which the parameter $Ao$ and thus the amino acid composition of the protein was determined also improved as the number of hydrolysis intervals increased. This was not true, however, when the number of hydrolysis intervals increased from 5 to 10 intervals (series II) but without the longer 141 hour hydrolysis (series III). The amino acid recoveries were reduced considerably when series II was used. This indicates that increasing the number of hydrolysis intervals does not guarantee an improvement in the accuracy with which the amino acid composition of the protein is determined.

Altering the range of intervals appears to have a substantial influence on the final estimates of $Ao$, $h$ and $l$. The unrealistically large estimates of $h$ for tyrosine, phenylalanine, leucine and lysine determined using only 5 hydrolysis intervals were the result of the first hydrolysis interval (4 hours) correspondng to the point where these particular amino acids had already reached or were very close to their maximum yield. The non-linear least squares model interpreted the data accordingly, designating a large $h$, indicating almost immediate hydrolysis of the amino acids from the protein-bound form. Increasing the number of hydrolysis intervals prior to the maximum point on the hydrolysis curve improved the accuracy of estimating $h$. Increasing the number of hydrolysis intervals also improved the estimation of $l$ as indicated by a reduction in the ASE. The importance of including an hydrolysis interval of considerable length (141 hours) is evident when the estimates of $h$ and $l$ derived using either series II (2-60 hours) or series III (2-141 hours) are compared (Tables 4.1 and 4.2). Given that the number of hydrolysis times before, at, and immediately after the maximum point on the hydrolysis curve had not changed, reducing the range of hydrolysis intervals only resulted in small changes in estimates of $h$. The estimation of $l$ differed considerably, however, with notably more zero estimates for some amino acids and higher estimates of $l$ for other amino acids when series II was used. This suggests that in addition to the importance of making several measurements before the hydrolysis curve reaches

a maximum, several longer hydrolysis intervals are necessary to provide an accurate estimate of *l*.

The sulphur amino acids are not routinely corrected for hydrolytic losses. Kohler and Palter (1967) observed significant losses of cysteine during acid hydrolysis, but stated that cysteic acid, by oxidising the protein prior to hydrolysis, was acid stable. Robel and Crane (1972) demonstrated that methionine, determined after acid hydrolysis without prior oxidation, exhibited a small loss rate. They did not investigate cysteine. In the present study, methionine and cysteine were analysed after performic acid oxidation and acid hydrolysis. Methionine sulphone had a much smaller loss rate (0.0001) compared to that reported for methionine (0.0008) by Robel and Crane (1972). Methionine that has not undergone prior oxidative treatment may also be lost through oxidation particularly if de-gassing of the sample is incomplete (Finley, 1985) and it is possible that the loss rate for methionine reported by Robel and Crane (1972) reflected this. The hydrolysis rate for cysteic acid was similar to that shown for valine and isoleucine, indicating that peptide bonds involving cysteic acid are difficult to cleave. The loss rate observed for cysteic acid was almost double that observed for serine. The low hydrolysis rate and high loss rate exhibited by cysteic acid demonstrates that, because of the simultaneous yield and decay process, simple extrapolation of points on an hydrolysis curve will lead to inaccurate determination of the cysteine in a protein. Recoveries of 90% or less for cysteic acid reported in the literature (Sittampalam et al., 1988) may be due to the failure to correct for this high loss rate for cysteic acid.

In the present study, the inadequacy of the conventional 24 hour measurement was obvious, particularly for glycine, serine, threonine, isoleucine and cysteic acid, where recoveries were all 90% or less. It is likely, that in addition to the error associated with a failure to correct for any losses of amino acids during hydrolysis, analysing duplicate samples only may introduce a sizable error. Estimation of the parameters *h*, *l* and *Ao* that provide the best-fit equation for the hydrolysis curve will reduce the error involved in determining the amino acid composition of a protein by both correcting for amino acid losses and by reducing the error associated with the points on the hydrolysis curve. This latter point is supported by the high recovery of amino acids in the present study (*Ao* for series III and IV compared to protein sequence data). This indicates that although precision at each hydrolysis interval may have been limited by the number of replicates, this was compensated for by an increase in the number of hydrolysis times. If an accurate estimate of the amino acid

composition of a protein is required, then it would appear that corrections for losses of amino acids during hydrolysis should be applied to a wider range of amino acids than is conventionally the case. Although Finley (1985) recommended that just 2 hydrolysis intervals be taken, with extrapolation to time zero for amino acids such as serine and threonine and to the maximum for amino acids such as valine and isoleucine, this method has limitations. The highest degree of precision and accuracy would be achieved when many replicates and hydrolysis times, respectively, are used to obtain data that can be fitted to a non-linear model to determine the amino acid composition of the protein. To obtain a cost-effective routine assay, however, the number of samples needs to be restricted. Duplicate sampling, although associated with a reduction in the precision of measurement at each hydrolysis interval, would allow more hydrolysis times to be taken. Assuming that the variation between the duplicates within each hydrolysis time can be minimised through careful sample preparation and hydrolysis techniques, the most accurate estimates of $h$, $l$ and $Ao$ will be obtained when many hydrolysis intervals are used. In the present study reducing the number of hydrolysis times to 10 (series III, range 2-141 hours) lowered the amino acid recoveries by less than 1% when compared to the amino acid composition determined using all 18 hydrolysis intervals. Measuring the amino acid yield in duplicate samples over 10 different hydrolysis intervals appears to be an appropriate alternative to the method proposed by Robel and Crane (1972), provided the range of hydrolysis intervals includes several times prior to the anticipated maximum point on the hydrolysis curve, and an hydrolysis interval greater than 100 hours.

The non-linear least squares fitting of the model to a series of different hydrolysis times can provide estimates of $h$ and $l$ for each amino acid in a particular protein. Subsequent analysis of the amino acid concentration of a protein hydrolysate taken at any given hydrolysis interval can be used to determine the amino acid composition of that protein by back-solution of the model to derive $Ao$. Back-solution of the mean of more than two replicates (for example, quadruplicates) is recommended to improve the precision of the final estimate. To this end, routine correction can be made for the losses of amino acids that occur during acid hydrolysis.

More research is needed to determine whether estimates of $h$ and $l$ vary for different types of protein. The suitability of this technique for protein mixtures and the influence of contaminants such as carbohydrates and fat on the loss rates observed during acid hydrolysis also need to be investigated.

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## APPENDIX 4A

The commands and partial derivatives needed to execute a non-linear least squares regression (*nlin* procedure, SAS, 1985) of a non-linear model to estimate the parameters *h*, *l* and *Ao* for an amino acid in a purified protein.

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**proc nlin** method = marquardt;

parms *Ao* = 2 *h* = 1 *l* = 0.1;

bounds *Ao*>0, *h*>0, *l*>=0;

$$\text{Model AA} = \frac{Ao\ h}{h - l}\left(e^{-lt} - e^{-ht}\right)$$

$$\text{der.}Ao = \left(\frac{h}{h - l}\right)\left(e^{-lt} - e^{-ht}\right)$$

$$\text{der.}h = \frac{\left(e^{-ht} - e^{-lt}\right) Ao\ l}{(h - l)^2} + \frac{t\ e^{-ht}\ Ao\ h}{(h - l)}$$

$$\text{der.}l = \frac{\left(e^{-lt} - e^{-ht}\right) Ao\ h}{(h - l)^2} - \frac{t\ e^{-lt}\ Ao\ h}{(h - l)}$$

run;

---

# CHAPTER 5

# CORRECTION FOR AMINO ACID LOSS DURING ACID HYDROLYSIS.

# II. LOSSES IN A PROTEIN MIXTURE CONTAINING FREE AMINO ACIDS

*Submitted - Analytical Biochemistry*

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Biological samples such as human milk contain not only a mixture of proteins but also amino acids in free form. Application of the model described in chapter 4 may not be appropriate for such a sample. The aim of the work described in this chapter was to determine whether the mathematical model needed to be modified to take into account free amino acids present in a sample prior to acid hydrolysis.

## 5.1 ABSTRACT

The simultaneous processes of amino acid yield and decay that occur during the hydrolysis of a purified protein prior to amino acid analysis can be described by a compartmental model with parameters for the hydrolysis (*h*) and loss (*l*) rates specific to each amino acid in a protein (**Model I**). Biological samples, however, often contain a mixture of different proteins, and amino acids in free form. Modification of the model is required, therefore, to allow for the effect of the presence of free amino acids prior to hydrolysis (**Model II**). The amino acid composition (*Ao*) of the sample prior to hydrolysis can be determined by non-linear regression of data derived from multiple hydrolysis intervals. In the present study a biological sample (human milk) was hydrolysed (4 replicates/hydrolysis interval) in acid using 23 hydrolysis intervals (range, 0-120 hours). The compartmental model parameters *Ao*, *h* and *l* were estimated and the final amino acid compositions were determined, using either **Model I** or **Model II**, and were compared. Most of the amino acids underwent some degree of loss during hydrolysis. Of particular note was the loss rate for cysteic acid, which was almost three times that shown for serine, which is commonly regarded as an acid-labile amino acid. When the conventional 24 hour hydrolysis method and model regression were used to determined the amino acid composition of the sample, there were considerable differences between the estimates for some of the amino acids previously thought to be acid-stable (24 hour vs **Model II**-predicted (μmol/g defatted freeze-dry. matter): PRO, 70 vs 88; ASP 70 vs 78; LEU 73 vs 83). Correction for losses of amino acids during hydrolysis is necessary if an accurate estimate of amino acid composition is required. Using **Model I** to analyse data obtained from hydrolysis of a sample containing protein and free amino acids will not lead to the introduction of any large bias in the determination of amino acid composition. The refined model (**Model II**), however, is more accurate for application whereby a sample contains both protein-bound and free amino acids.

## 5.2 INTRODUCTION

During the hydrolysis of a protein, there are simultaneous processes that release free amino acids from their protein-bound form and degrade amino acids in the free form. These processes can be described by a compartmental analysis model (Matis and Hartley, 1971) with three distinct compartments (states). State A represents the amount of an amino acid in protein-bound form. As amino acids are cleaved from the protein they shift to State B where detection occurs. Susceptible amino acids that are converted to unidentifiable compounds or are simply degraded during acid hydrolysis, shift to State C and are no longer detectable. The amount of amino acid present in state B at time **t** is determined by the amount of amino acid present prior to hydrolysis (t=0), the rate of release of that amino acid during hydrolysis (from State A to B) and any subsequent loss of the amino acid (from State B to C). This dynamic relationship can be described by the following equation (**Model I**):

$$B(t) = \frac{Ao\ h}{h - l}\left(e^{-lt} - e^{-ht}\right) + \epsilon \qquad (1)$$

where,

| | | |
|---|---|---|
| B(t) | = | amino acid measured at time t. |
| *Ao* | = | the quantity of amino acids in the protein prior to hydrolysis, ie. at t = 0. |
| *h* | = | the rate at which the protein-bound amino acid is hydrolysed to a free, and measurable form. |
| *l* | = | the rate at which the amino acid is degraded to an undetectable form. |
| ε | = | random residual error that accounts for measurement error among other factors. |

This model assumes that the rate of destruction of labile amino acids while still peptide-bound is minor (Robel and Crane, 1972). It is also assumed that the hydrolysis rate (*h*), expressed as a proportion of the protein-bound amino acid per hour, and the loss rate (*l*), expressed as a proportion of the detectable amino acids per hour, are constant fractions of the amounts of amino acid in State A and B, respectively. Estimates of *h*, *l*, and *Ao* for a particular protein can be derived using non-linear least-

squares regression of a series of B(t) values determined at different hydrolysis intervals (Robel and Crane, 1972). The model is applicable to all amino acids regardless of the rate of hydrolysis or loss exhibited by each individual amino acid.

This approach has been used previously by Robel and Crane (1972), and in chapter 4, to determine the amino acid composition of a purified protein. In many instances, particularly when dealing with biological substances, the sample to be analysed will contain a mixture of proteins. In addition, some of the amino acids in the initial sample may be in free form. If multiple hydrolysis intervals are used to determine the amino acid composition of this type of sample, then it must be assumed that the compartmental model is applicable to a protein mixture, with the parameter $h$ pertaining to a particular amino acid remaining constant regardless of which type of protein is being hydrolysed. As the loss rate is given as a fraction of the amount of amino acids in State B, it should remain constant regardless of the source of free amino acids. According to Model I, the only source of free amino acids is from hydrolysis of the protein. Therefore the presence of free amino acids at the start of hydrolysis would upwardly bias estimates of the hydrolysis rate, $h$. The size of this bias would depend on the first hydrolysis interval and the quantity of free amino acid at time zero. Therefore, a modification of Model I is proposed, to allow for the effect of the presence of free amino acids at time zero.

Assuming that free amino acids shift to state C at a constant rate, the destruction of these amino acids over time can be described by the following equation:

$$B(t) = Bo e^{-lt} + \epsilon \qquad (2)$$

where $Bo$ = the amount of free amino acid present prior to the start of hydrolysis.

The determined amino acid concentrations in hydrolysates during hydrolysis is, therefore, the sum of the concentrations resulting from the processess described by both equation 1 (release of protein-bound amino acids and subsequent loss of free amino acids) and equation 2 (loss of free amino acids present at time zero).

While equation 1 may be appropriate for use with a purified protein, a new model (equation 3) derived by combining equations 1 and 2 more accurately describes the hydrolysis of a sample containing both protein and free amino acids at time zero.

$$B(t) = \frac{Ao\ h}{h - l}(e^{-lt} - e^{-ht}) + Boe^{-lt} + \epsilon \qquad (3)$$

The amino acid composition of the sample is determined by summation of *Ao* (the amount of protein-bound amino acid at time zero) and *Bo* (the amount of free amino acid at time zero).

In the present study a biological sample (human milk), which was a mixture of different proteins and free amino acids, was hydrolysed in acid over a range (1-120 hours) of hydrolysis intervals. The compartmental model parameters *Ao*, *h* and *l* were estimated and the final amino acid compositions determined, using either equation 1 (**Model I**) or equation 3 (**Model II**), were compared.

## 5.3 EXPERIMENTAL

### 5.3.1 **Milk collection**

Approval was obtained from the Massey University Human Ethics Committee for the collection of human milk samples from women in their second to fourth months of lactation. The method of milk collection and sampling has been described in detail elsewhere (refer chapter 6).

### 5.3.2 **Chemical Analysis**

The representative sample of pooled human milk was freeze-dried, then defatted with light petroleum ether (b.p. 40°-60°C) in a Soxhlet apparatus prior to amino acid analysis. High fat contents have been shown to interfere with the determination of amino acids (Finley, 1985).

Samples (5-7 mg) of defatted freeze-dried human milk were accurately weighed into 84 x 10 ml acid-washed pyrolysed (650°C, overnight) glass test tubes each containing 1 ml of glass-distilled 6 M HCl (with 1% phenol w/v). The tubes were sealed under vacuum and hydrolysed in quadruplicate at 110 ± 2°C for either 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 60 or 120 hours. For determination of *Bo*, 1 ml of 6 M HCl was added to 4 additional test tubes each containing 5-7 mg of defatted freeze-dried human milk. The acid in these latter samples was immediately removed under vacuum (Automatic Speedvac Concentrator AS290, Savant Instruments Inc, Farmingdale, NY). After the specified hydrolysis

intervals the samples were dried down by centrifugation under vacuum. Loading buffer (1 ml of 0.2 M sodium citrate, pH 2.2) was added to each of the 88 dried samples. Samples that had been hydrolysed for less than 6 hours were deproteinised by ultrafiltration using a micropartition system (molecular exclusion limit: 10,000 Da, Amicon Division, Grace & Co, Danvers, MA, USA). After dissolving the amino acids in loading buffer the samples were loaded onto a Pharmacia LKB - Alpha Plus Amino Acid Analyser (Cambridge, England). The amino acids were separated by ion-exchange chromatography and detected following reaction with ninhydrin.

Samples of the defatted freeze-dried human milk were also analysed for cysteine and methionine. Freshly prepared performic acid (1 part 30% $H_2O_2$ to 9 parts 88% formic acid) was added to 88 acid-washed, pyrolysed test tubes (2ml/tube) each containing 5-7 mg of defatted freeze-dried human milk. The tubes were left at 0°C for 16 hours, after which time 300 µl of 48% HBr was added to each tube. The samples were dried under vacuum and 1 ml of 6 M HCl (with 1% phenol w/v) was added to each tube. To determine the cysteine and methionine present at time zero (*Bo*), the acid in 4 of the test tubes was immediately dried down under vacuum. The remaining 84 samples were hydrolysed under the same conditions and for the same intervals as described above. After hydrolysis and acid removal, 2 ml of loading buffer (sodium citrate) was added to each of the 88 tubes. As before the samples that had been hydrolysed for less than 6 hours were filtered. The samples were loaded on to an ion-exchange HPLC System (Millipore Corporation, Waters Chromatography Division, Milford, MA, USA), utilising post-column *O*-phthalaldehyde derivitisation and fluorescence detection. Cysteine and methionine were detected as cysteic acid and methionine sulphone, respectively. Tryptophan, being destroyed during acid hydrolysis, was not determined.

### 5.3.3 **Data Analysis**

The quadruplicate analyses for each hydrolysis interval were averaged to provide B(t) values. For either **Model I** or **Model II**, the best fit equation as defined by the parameters $h$, $l$ and $Ao$ was computed for each amino acid using a non-linear least squares regression procedure conducted within a defined parameter space ($Ao>0$, $h>0$ and $l>=0$). The Marquardt method was used for analysis. The instructions for the non-linear regression procedure (SAS, 1985) including the partial derivatives of the non-linear model, are given in Appendices 5a and 5b (pages 157-158) for **Model I** and

**Model II**, respectively. It is assumed that the estimates of the model parameters are asymptotically normal.

## 5.4 RESULTS AND DISCUSSION

The limitations of hydrolysing a protein in acid for a single time period to determine the amino acid composition are well accepted. Corrections need to be made for the delayed hydrolysis of some amino acids, and for losses that will occur with acid labile amino acids (Davies and Thomas, 1973; Blackburn, 1978; Finley, 1985).

In the present study the amino acid composition of a biological sample (human milk) containing several proteins and free amino acids was determined by non-linear regression of amino acid concentration data measured at multiple hydrolysis times. Four replicate samples were analysed for each hydrolysis interval and the overall mean coefficients of variation (between the quadruplicates at each hydrolysis interval) were 12.3% and 7.2%, for the ninhydrin detected amino acids and the sulphur amino acids, respectively.

The accuracy with which amino acid composition can be determined is a function of the number of intervals used and the number of replicates per interval. It is possible to obtain an acceptable degree of accuracy in amino acid determination of a purified protein using 10 hydrolysis intervals (refer chapter 4). The number of hydrolysis intervals used in the present study, however, was increased to 23 because it was not known to what extent the variability of the data would be affected by hydrolysing a sample containing not only a mixture of proteins, peptides and free amino acids but also a range of other compounds.

Due to inadequate chromatograph peak resolution, phenylalanine concentrations were unable to be determined following ninhydrin detection. However, it was possible to determine phenylalanine after *O*-phthalaldehyde derivitisation and fluorescence detection. Therefore the phenylalanine results presented were determined after performic acid oxidation and acid hydrolysis. As phenylalanine detected after performic acid oxidation of a standard egg-white lysozyme sample showed only a 90% recovery (AJ Darragh, unpublished), the phenylalanine results should be interpreted with caution. The concentrations of free amino acids measured in the sample at time zero (B(t) at t=0, ie. *Bo*) are given in Table 5.1. Glutamic acid and cysteine were the amino acids in free form present prior to acid hydrolysis in the greatest quantities.

**Table 5.1.**

*Free amino acids* present in mature human milk+*

| Amino Acid | *Bo#* | Amino Acid | *Bo#* |
|---|---|---|---|
| **Glutamic Acid** | 11.9 ± 0.12 | **Leucine** | 1.0 ± 0.13 |
| **Glycine** | 3.1 ± 0.14 | **Tyrosine** | 1.6 ± 0.04 |
| **Proline** | 1.4 ± 0.05 | **Phenylalanine** | 0§ |
| **Serine** | 4.0 ± 0.11 | **Histidine** | 0.6 ± 0.26 |
| **Threonine** | 3.8 ± 0.07 | **Lysine** | 1.6 ± 0.48 |
| **Aspartic Acid** | 2.8 ± 0.35 | **Arginine** | 1.6 ± 0.71 |
| **Alanine** | 3.1 ± 0.16 | **Methionine** | 0§ |
| **Valine** | 3.0 ± 0.13 | **Cysteic Acid** | 8.4 ± 0.13 |
| **Isoleucine** | 1.0 ± 0.10 | | |

* µmol/g defatted freeze-dried matter.

\+ Pooled representative sample subsampled from milk collected from 20 mothers in their 2-4 months of lactation.

\# Determined after addition of 6 M HCl followed by removal of acid under vacuum.

§ Not detected.

Plots of amino acid yield in human milk for the multiple hydrolysis intervals are given in Figures 5.1 and 5.2, respectively. For most of the amino acids, the maximum point on the hydrolysis curve occurred before 15 hours, with the exception of threonine, valine and isoleucine where maxima occurred between 16 and 18 hours. Of particular note was the rapid hydrolysis of cysteic acid, where the maximum was reached after 1 hour. Most of the amino acids showed a distinct decline over time.

Estimates of $h$ and $l$ derived using either **Model** I or II are given in Table 5.2. Based on the reasoning developed in the present introduction, **Model** II more accurately describes the actual processes of hydrolysis and loss in a sample containing both protein-bound and free amino acids. The **Model** II estimates can therefore be regarded as the basis for comparison with **Model** I. To this end, it appears that using **Model** I leads to an overestimation of $h$ and an underestimation of $l$, although not markedly so. Estimates of $h$ are likely to be overestimated using **Model** I because the

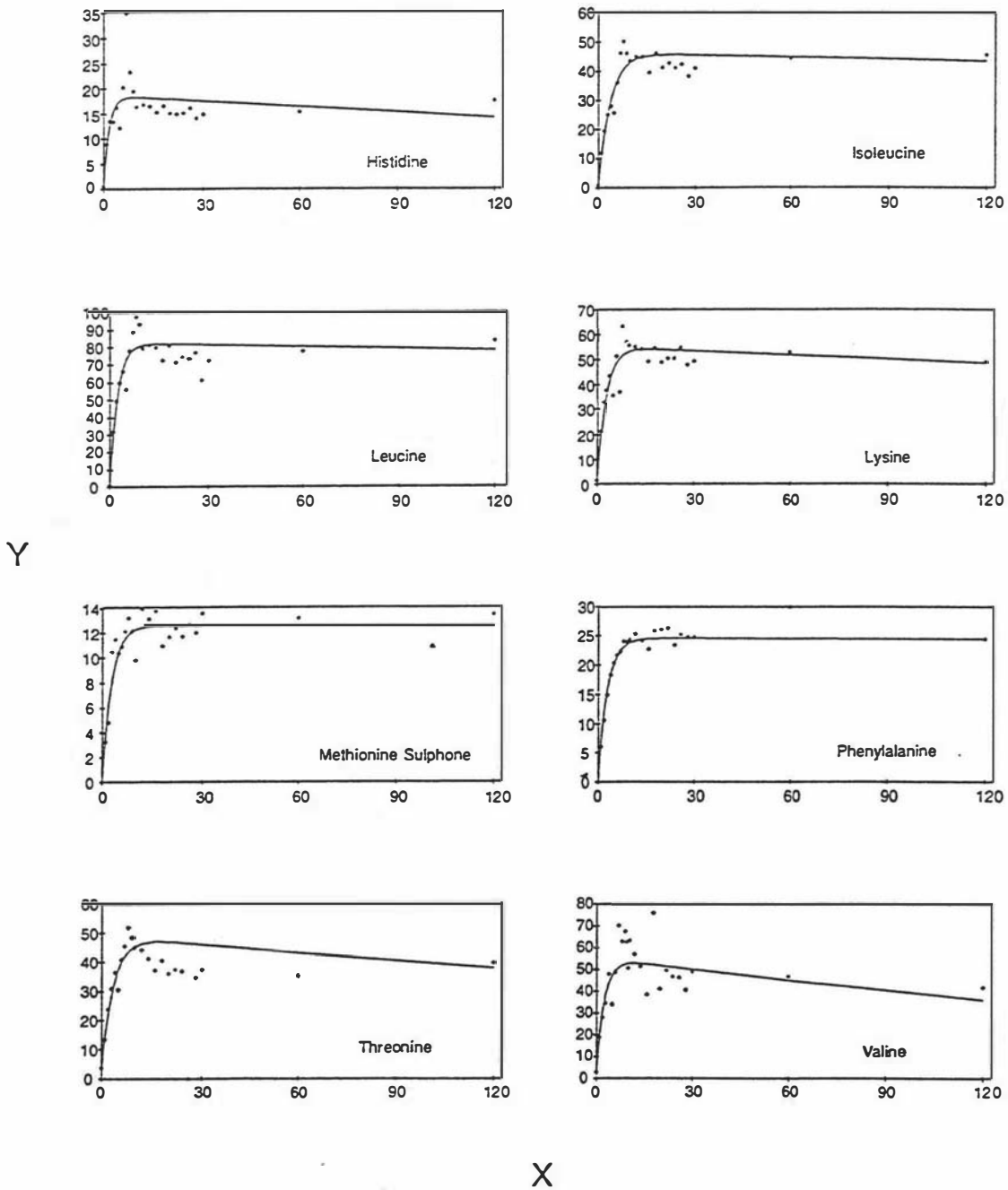


**Figure 5.1.**

Effect of hydrolysis time (X axis, hours) during amino acid analysis on the mean yield of amino acids (Y axis, μmoles of amino acid per g defatted freeze-dried matter) in human milk. The mean value of quadruplicate samples (♦) and the line of best fit determined using estimates of *h*, *l* and *Ao* pertaining to the data are plotted.

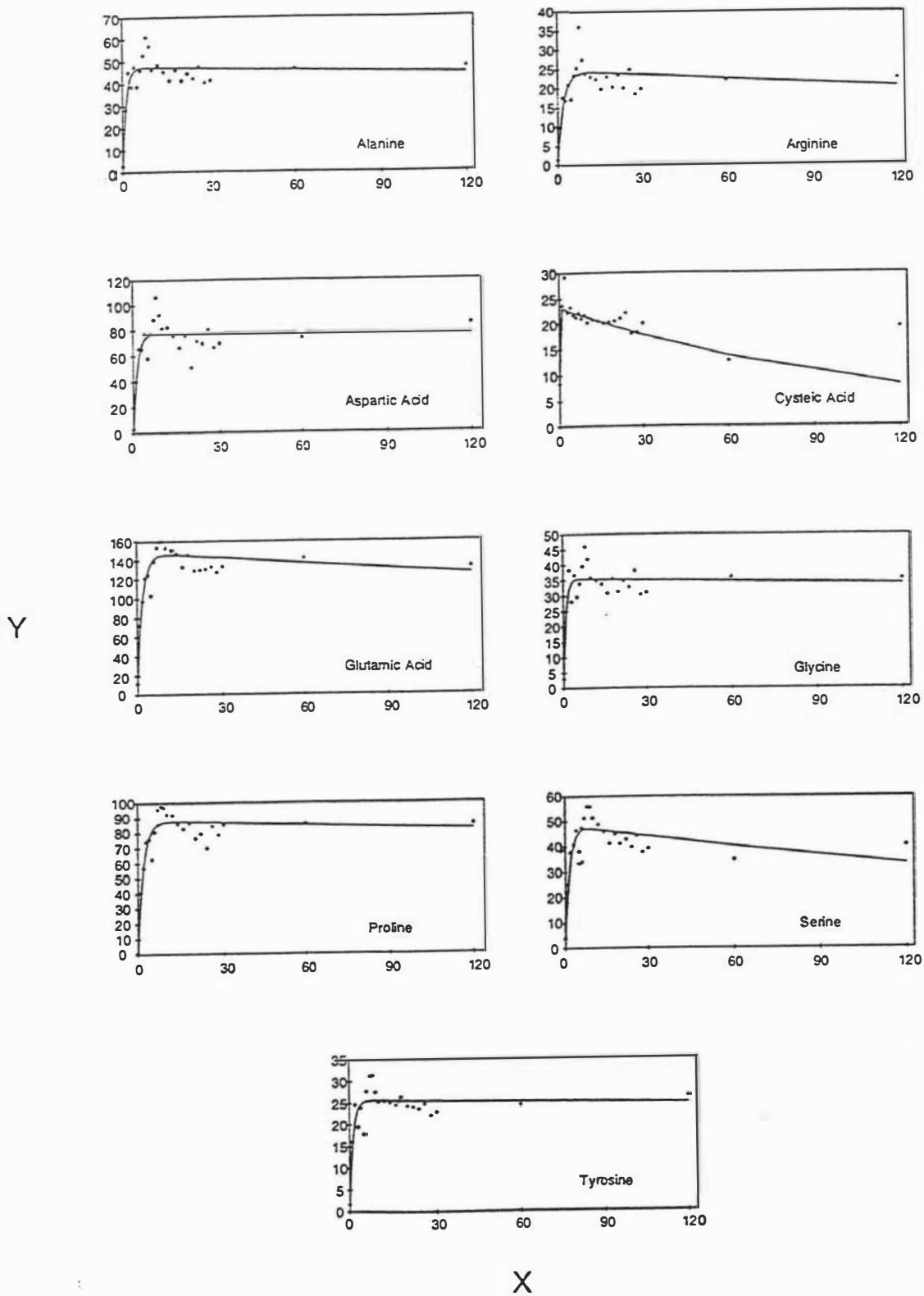


**Figure 5.2.**

Effect of hydrolysis time (X axis, hours) during amino acid analysis on the mean yield of amino acids (Y axis, μmoles of amino acid per g defatted freeze-dried matter) in human milk. The mean value of quadruplicate samples (♦) and the line of best fit determined using estimates of *h*, *l* and *Ao* pertaining to the data are plotted.

**Table 5.2.**

*Hydrolysis (h) and loss (l) rates (fractions of A* or B+ per hour, respectively) ± ASE# for amino acids in a human milk§ sample during acid hydrolysis determined by non-linear least squares regression for multiple hydrolysis intervals¶ using either a three-way compartmental model (I) or a four-way compartmental model (II)*

| | Model I | | Model II | |
|---|---|---|---|---|
| Amino Acid | *h* | *l* | *h* | *l* |
| **Glutamic Acid** | 0.571 ± 0.0960 | 0.0013 ± 0.00098 | 0.520 ± 0.0909 | 0.0014 ± 0.00096 |
| **Glycine** | 1.235 ± 0.4540 | 0.0004 ± 0.00132 | 1.120 ± 0.4309 | 0.0004 ± 0.00131 |
| **Proline** | 0.527 ± 0.0962 | 0.0006 ± 0.00104 | 0.517 ± 0.0952 | 0.0006 ± 0.00104 |
| **Serine** | 0.687 ± 0.1510 | 0.0031 ± 0.00136 | 0.623 ± 0.1431 | 0.0032 ± 0.00136 |
| **Threonine** | 0.313 ± 0.1681 | 0.0020 ± 0.00423 | 0.276 ± 0.1577 | 0.0021 ± 0.00433 |
| **Aspartic Acid** | 0.757 ± 0.2265 | 0.0001 ± 0.00138 | 0.729 ± 0.2237 | 0.0001 ± 0.00138 |
| **Alanine** | 0.890 ± 0.2639 | 0.0005 ± 0.00131 | 0.820 ± 0.2522 | 0.0006 ± 0.00131 |
| **Valine** | 0.434 ± 0.0688 | 0.0017 ± 0.00109 | 0.402 ± 0.0067 | 0.0018 ± 0.00110 |
| **Isoleucine** | 0.276 ± 0.0490 | 0.0005 ± 0.00128 | 0.269 ± 0.0488 | 0.0005 ± 0.00129 |
| **Leucine** | 0.443 ± 0.1042 | 0.0005 ± 0.00142 | 0.436 ± 0.1037 | 0.0005 ± 0.00142 |
| **Tyrosine** | 0.902 ± 0.2339 | 0.0003 ± 0.00111 | 0.829 ± 0.2197 | 0.0003 ± 0.00110 |
| **Phenylalanine** | 0.310 ± 0.0165 | 0.0003 ± 0.00037 | 0.310 ± 0.5381 | 0.0003 ± 0.00037 |
| **Histidine** | 0.608 ± 0.2672 | 0.0023 ± 0.00272 | 0.592 ± 0.2674 | 0.0023 ± 0.00272 |
| **Lysine** | 0.382 ± 0.0685 | 0.0001 ± 0.00121 | 0.367 ± 0.0665 | 0.0010 ± 0.00120 |
| **Arginine** | 0.507 ± 0.1453 | 0.0015 ± 0.00179 | 0.475 ± 0.1426 | 0.0015 ± 0.00180 |
| **Methionine°** | 0.726 ± 0.6710 | 0.0000 ± 0.00426 | 0.726 ± 0.6710 | 0.0000 ± 0.00426 |
| **Cysteine"** | 76.720 ± 0.0001 | 0.0088 ± 0.00634 | 14.220 ± 0.0001 | 0.0088 ± 0.00357 |

* Corresponds to the amount of protein-bound amino acids remaining during hydrolysis.
\+ Corresponds to the amount of amino acid in free form during hydrolysis.
\# Asymptotic Standard Error.
§ Pooled representative sample taken from milk collected from 20 mothers in their 2-4 months of lactation.
¶ 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 60 and 120 hours.
° Detected as Methionine sulphone.
" Detected as Cysteic Acid.

presence of free amino acids at time zero is indicative of hydrolysis in progress and this will need to be factored into the estimation of *h*. Likewise, the presence of free amino acids may result in an underestimation of the *l* rate based on early hydrolysis intervals. The large *h* value estimated for cysteic acid using either **Model I** or **II** is almost certainly due to the large amounts of cysteic acid in free form at time zero. A similar result was not found for glutamic acid, the other amino acid present in large amounts in free form prior to hydrolysis, because the amount of glutamic acid in free form at t=0 was small in proportion to the final concentration of glutamic acid in human milk. Valine and isoleucine have been identified as being difficult to cleave from their protein bonds during hydrolysis (Blackburn 1978). In the present study several other amino acids including threonine, lysine and phenylalanine also had relatively slow hydrolysis rates as indicated by the values for *h* given in Table 5.2.

From the present study, it would appear that, in addition to serine and threonine, which are well recognised as being acid labile (Finley, 1985), several other amino acids experience losses during acid hydrolysis. The high loss rate for cysteic acid deserves specific attention, as this amino acid shows a loss rate almost three times that of serine. In contrast, the other sulphur amino acid, methionine, when detected as methionine sulphone, shows a zero loss rate during acid hydrolysis.

The observation that threonine was the second slowest amino acid to cleave from its protein-bound form has particular relevance to the final determination of threonine in the sample, when the relatively high loss rate for threonine is also considered. Due to the process of simultaneous yield and decay, the determination of an amino acid exhibiting both a slow release and rapid loss rate after a single hydrolysis interval, or even after linear extrapolation would be inaccurate. As with threonine, valine has a slow release and relatively high loss rate which makes determination by non-linear regression essential.

The differences between the estimates of *h* and *l*, determined using either **Model I** or **II**, were small. These model parameters together with *Ao* are derived simultaneously during the regression procedure, however, and an over- or under-estimation of either *h* or *l* will influence the accuracy with which *Ao* is estimated. Estimates of the amino acid composition of the human milk sample determined from the **Model I** estimation of *Ao*, or from the summation of *Bo* and the **Model II** estimation of *Ao* are given in Table 5.3. As with the estimation of *h*, **Model I** consistently overestimated the amino acid composition of the human milk sample

**Table 5.3.**

*The amino acid composition* of a biological sample containing a mixture of proteins and free amino acids (human milk+) determined by either non-linear regression (Model I or Model II) for multiple hydrolysis intervals# or by the routine 24 hour acid hydrolysis*

| | Model I§ | Model II¶ | | |
|---|---|---|---|---|
| Amino Acid | *Ao* ± ASE° | *Ao* ± ASE | *Ao* + *Bo* | 24 Hr Hydrolysis= |
| **Glutamic Acid** | 149.0 ± 4.15 | 135.8 ± 4.13 | 147.7 | 131.1 ± 2.79 |
| **Glycine** | 36.5 ± 1.19 | 32.5 ± 1.19 | 35.6 | 32.9 ± 0.79 |
| **Proline** | 88.3 ± 2.64 | 86.9 ± 2.65 | 88.3 | 69.8 ± 10.07 |
| **Serine** | 49.0 ± 1.58 | 44.3 ± 1.59 | 48.3 | 39.9 ± 0.57 |
| **Threonine** | 49.1 ± 6.00 | 45.1 ± 6.32 | 48.9 | 36.9 ± 0.71 |
| **Aspartic Acid** | 78.1 ± 3.16 | 74.9 ± 3.18 | 77.7 | 69.7 ± 1.90 |
| **Alanine** | 48.4 ± 1.64 | 44.7 ± 1.65 | 47.8 | 42.2 ± 1.11 |
| **Valine** | 55.7 ± 3.18 | 52.2 ± 3.25 | 55.2 | 46.8 ± 3.86 |
| **Isoleucine** | 46.3 ± 1.98 | 45.2 ± 2.00 | 46.2 | 41.2 ± 1.44 |
| **Leucine** | 82.6 ± 3.59 | 81.5 ± 3.61 | 82.5 | 73.3 ± 2.00 |
| **Tyrosine** | 25.8 ± 0.86 | 24.1 ± 0.86 | 25.7 | 23.6 ± 0.72 |
| **Phenylalanine** | 24.6 ± 2.25 | 24.6 ± 2.21 | 24.6 | 23.4 ± 2.01 |
| **Histidine** | 18.8 ± 1.36 | 18.1 ± 1.37 | 18.7 | 15.2 ± 0.34 |
| **Lysine** | 54.9 ± 2.03 | 53.2 ± 2.05 | 54.8 | 50.4 ± 1.25 |
| **Arginine** | 24.6 ± 1.28 | 22.9 ± 1.30 | 24.5 | 20.2 ± 0.50 |
| **Methionine**** | 12.6 ± 0.48 | 12.6 ± 0.48 | 12.6 | 12.2 ± 0.67 |
| **Cysteine++** | 24.7 ± 1.73 | 14.9 ± 0.97 | 23.3 | 22.3 ± 2.10 |

* µmol/g defatted freeze-dried matter.

\+ Pooled representative sample taken from milk collected from 20 mothers in their 2-4 months of lactation.

\# 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 60 and 120 hours.

§ Model I is a non-linear three compartmental model with a parameter *Ao* which estimates the amino acid composition of the sample.

¶ Model II is a non-linear four compartmental model with a parameter *Ao* which estimates the amino acid composition of the protein in the sample. *Ao* is then added to *Bo* (the free amino acids measured at time zero) to give the final estimate of the amino acid composition of the sample.

° Asymptotic Standard Error.

= Mean value (± SE) of quadruplicates.

** Detected as Methionine sulphone.

++ Detected as Cysteic Acid.

(assuming that the summation of *Bo* and *Ao* (**Model** II) provides the more accurate estimate), although again the differences were small. The mean amino acid composition of quadruplicate samples determined after 24 hours hydrolysis is also given in Table 5.3. The need to correct losses of amino acids is strikingly clear when these values are compared with the amino acid compositions determined using both **Models** I and II.

When *Bo* is zero as would be the case with a sample containing a purified protein, using either **Model** I or II to analyze the data obtained after multiple hydrolysis of the sample will result in the same estimates of *h*, *l* and *Ao* regardless of which model is used because the term involving *Bo* in equation 3 becomes zero. Likewise, given the small differences observed between the estimates of *h*, *l* and final amino acid composition of human milk determined using either **Model** I or II, it would appear that it is not crucial which model is chosen to analyze the data obtained from hydrolysis of a sample containing both protein-bound and free amino acids. In the present study, however, the comparison of **Model** I and II involved the analysis of one set of data only. For a full comparison, the two models would need to be applied to several mixtures with different hydrolytic properties.

To do this, 3 theoretical sets of data (based on a protein containing 10 units of a given amino acid, and using hydrolysis and loss rates of a magnitude assumed to occur in protein mixtures) were analysed using either **Model** I or II. Each set of data consisted of 10 simulated subsets derived by entering defined values for the parameters *h*, *l*, *Ao* and *Bo* into equation 3 to obtain a series of B(t) values from t=0 to t=100 (Set 1: *Ao*=9, *Bo*=1, *h*=0.1 and *l*=0.005; Set 2: *Ao*=9, *Bo*=1, *h*=1 and *l*=0.005; Set 3: *Ao*=9, *Bo*=1, *h*=10 and *l*=0.005). A random error component (sampled from a normal distrubtion with mean=0 and standard deviation=0.25) was added to each B(t) value. The estimates of *h* and *l* and the amino acid composition determined based on *Ao* (**Model** I) and the summation of *Ao* and *Bo* (**Model** II) are given in Table 5.4. The greatest differences in the estimates of *h*, *l* and *Ao* occurred when the hydrolysis rate was small ($h = 0.1$). In general, however, using **Model** I to analyze data obtained from hydrolysis of a sample containing protein and free amino acids will not lead to the introduction of any large bias in the determination of amino acid composition. It remains more logical, however, to analyze each type of data with the most appropriate model. In the situation where a sample contains both protein-bound and free amino acids, **Model** II

**Table 5.4.**

*Estimates (mean ± SE) of amino acid composition (A), h and l (mean* ± SE) derived using either Model I+ or Model II# to analyse three simulated sets of data*

| Simulated Data Set¶ | Model | Parameters§ | | |
|---|---|---|---|---|
| | | A° | *h* | *l* |
| 1 | I | 9.52 ± 0.451 | 0.149 ± 0.0265 | 0.0041 ± 0.00114 |
| | II | 10.21 ± 0.546 | 0.113 ± 0.0259 | 0.0050 ± 0.00120 |
| 2 | I | 9.89 ± 0.302 | 1.490 ± 0.7459 | 0.0048 ± 0.00099 |
| | II | 9.90 ± 0.305 | 1.439 ± 0.7473 | 0.0048 ± 0.00100 |
| 3 | I | 10.02 ± 0.217 | 38.020 ± 67.5491 | 0.0047 ± 0.00084 |
| | II | 10.02 ± 0.217 | 35.849 ± 63.3648 | 0.0047 ± 0.00084 |

* Mean value of 10 data sets within each group. Each data set was derived using Model II with a random error component added to each value.

\+ Model I is a three-way compartmental model described by Robel and Crane (1972).

\# Model II is a four-way compartmental model which allows for free amino acids at time zero.

§ *h* and *l* are expressed as units of amino acid, fraction of State A (amount of amino acids in protein-bound form) per hour and fractions of State B (amount of amino acid in free form) and Bo (amount of amino acid in free form at t=0) per hour, respectively.

¶ Each group had 10 data sets derived using specific *Ao*, *h* and *l* parameters, with an error component added (Group 1: *Ao* = 9, *h* = 0.1, *l* = 0.005; Group 2: *Ao* = 9, *h* = 1, *l* = 0.005; Group 3: *Ao* = 9, *h* = 10, *l* = 0.005; For all Groups *Bo* = 1).

° A = *Ao* for Model I and *Ao* + *Bo* for Model II.

should be used.

From the present study it would appear that during acid hydrolysis losses occur for most of the amino acids (except methionine) in human milk. Correction for these losses is necessary if an accurate estimate of amino acid composition is required. The estimates of *h* and *l* determined in the present study can be used to correct the amino acid composition of a single hydrolysate measured at time t by rearranging equation 3 to obtain an estimate of *Ao* (see equation 4).

$$Ao = \frac{(B(t) - (Bo\, e^{-lt}))\,(h - l)}{(e^{-lt} - e^{-ht})\,h} \qquad (4)$$

This is only possible, however, if conditions of hydrolysis are identical to those used in the present study. To improve the accuracy with which *Ao* is determined, several

replicates should be used for the particular hydrolysis interval chosen. Alternatively, accuracy may be improved by using B(t) values from several different hydrolysis intervals to calculate numerous estimates of *Ao* which can then be averaged. Determining the amino acid composition relies on the summation of *Ao* and *Bo*. It is important, therefore, that *Bo* is accurately determined. This is particularly important for those amino acids where a significant proportion of the total amount is in free form at time zero (eg. cysteine).

## 5.5 REFERENCES

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## APPENDIX 5a

The commands and partial derivatives needed to execute a non-linear least squares regression (*nlin* procedure, SAS, 1985) of a non-linear model to estimate the parameters *h*, *l* and *Ao* for an amino acid in a purified protein.

---

### MODEL I

**proc nlin** method = marquardt;

parms *Ao* = 2 *h* = 1 *l* = 0.1;

bounds *Ao*>0, *h*>0, *l*>=0;

$$\text{Model AA} = \frac{Ao\ h}{h - l}\left(e^{-lt} - e^{-ht}\right)$$

$$\text{der.}Ao = \left(\frac{h}{h - l}\right)\left(e^{-lt} - e^{-ht}\right)$$

$$\text{der.}h = \frac{\left(e^{-ht} - e^{-lt}\right)\ Ao\ l}{(h - l)^2} + \frac{t\ e^{-ht}\ Ao\ h}{(h - l)}$$

$$\text{der.}l = \frac{\left(e^{-lt} - e^{-ht}\right)\ Ao\ h}{(h - l)^2} - \frac{t\ e^{-lt}\ Ao\ h}{(h - l)}$$

run;

---

## APPENDIX 5b

The commands and partial derivatives needed to execute a non-linear least squares regression (*nlin* procedure, SAS, 1985) of a non-linear model to estimate the parameters *h*, *l* and *Ao* for an amino acid in a sample containing both protein-bound and free amino acids.

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### MODEL II

**proc nlin** method = marquardt;

parms $Ao = 2 \quad h = 1 \quad l = 0.1$;

bounds $Ao > 0, h > 0, l >= 0$;

$$\text{Model } AA = \frac{Ao\ h}{h - l}(e^{-lt} - e^{-ht}) + Bo(e^{-lt})$$

$$\text{der.}Ao = \left(\frac{h}{h - l}\right)(e^{-lt} - e^{-ht})$$

$$\text{der.}h = \frac{(e^{-ht} - e^{-lt})\ Ao\ l}{(h - l)^2} + \frac{t\ e^{-ht}\ Ao\ h}{(h - l)}$$

$$\text{der.}l = \frac{(e^{-lt} - e^{-ht})\ Ao\ h}{(h - l)^2} - \frac{t\ e^{-lt}\ Ao\ h}{(h - l)} - t\ Bo\ e^{-lt}$$

run; .

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# CHAPTER 6

# THE AMINO ACID COMPOSITION OF HUMAN MILK CORRECTED FOR AMINO ACID DIGESTIBILITY

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The main objective of this thesis was to determine the digestible amino acid composition of human milk, using the piglet as a model animal for the human infant. To this end, evidence was obtained to support the use of the piglet as a model animal (chapter 2) and the use of the ileal digestibility assay in piglets (chapter 3). A method for accurately determining the amino acid composition of human milk has also been established (chapters 4 and 5). In the work described in this chapter, the amino acid composition of human milk was determined and corrected for digestibility using true ileal amino acid digestibility coefficients determined in piglets fed human milk.

## 6.1 ABSTRACT

Human milk was collected from women in their 10th-14th weeks of lactation, and was analysed for amino acids. Corrections were made for losses of amino acids which occur during acid hydrolysis, using a non-linear mathematical model that describes the simultaneous processes of amino acid yield and decay. The mean amino acid composition of the human milk was found to be similar to previously reported estimates, though the cysteine content of the human milk in the present study was 20% higher than the average literature estimate. True (corrected for endogenous amino acid excretions) ileal amino acid digestibility of human milk was determined using the three-week-old piglet as a model animal for the human infant. The piglets were given either human milk (n=6) or a protein-free diet (n=6) for 6 days. Chromic oxide was added as an indigestible marker, to both the human milk and protein-free diet. At the end of the 6 day experimental period the piglets were anaesthetised and samples of digesta were removed from the terminal 40 cm of each piglet's ileum. Endogenous ileal excretions of amino acids were determined in piglets fed the protein-free diet. The mean true digestibilities of total nitrogen and amino acid nitrogen were 88% and 95%, respectively. The mean true ileal digestibility of the non-amino acid nitrogen fraction in human milk, when calculated by difference was only 50%. The mean true digestibilities of the amino acids in human milk ranged from 81-101% with threonine (86%) being the least digestible essential amino acid. When the true ileal digestibility values were used to correct the amino acid composition of human milk, the pattern of digestible amino acids in human milk was different compared to the currently recommended dietary pattern of amino acids for the infant.

## 6.2 INTRODUCTION

The amino acid composition of human milk is widely accepted as the standard upon which to base the protein and amino acid requirements of infants (Committee of Nutrition 1985, FAO/WHO/UNU 1985). There are two potential inaccuracies, however, with present estimates of the profile of amino acids in human milk. Firstly, corrections for losses of amino acids which occur during the acid hydrolysis of milk samples prior to amino acid detection, if made at all, are usually limited to serine and threonine. It has been demonstrated by Robel and Crane (1972), and in chapters 4 and 5, that for absolute accuracy corrections for other amino acids are also necessary. Secondly, it is assumed that the amino acids in human milk are completely digested. This may not be correct, as there are a number of proteins present in human milk the nutritional role of which is uncertain. Lactoferrin and secretory IgA (sIgA), comprising approximately 20% and 10% of the total protein in human milk, respectively (van Woelderen, 1987), are involved in immune protection of the gastrointestinal tract (Lönnerdal, 1985). Significant amounts of these proteins have been identified in the faeces of breast-fed infants (Ogra *et al.* 1977; Davidson and Lönnerdal, 1987; Prentice *et al.* 1989). Although sIgA and lactoferrin, presumably of endogenous origin, have also been identified in the faeces of formula-fed infants (Haneberg and Tonder, 1973; Spik *et al.* 1982), the considerably higher amounts found in the faeces of breast-fed infants would suggest that sIgA and lactoferrin partially survive proteolysis in the gut. This has direct nutritional implications, with the gross amino acid composition of human milk not necessarily reflecting the amino acid requirements of the infant.

Estimates of the digestible protein in human milk, based on the amounts of immune proteins detected in the breast-fed infant's faeces, range from 70% of total protein (Hambraeus *et al.* 1984) to 90-95% of total protein (Davidson and Lönnerdal, 1987). However, there is considerable microbial degradation of protein in the large intestine (McNeil, 1988) and amino acids do not appear to be absorbed in human colonic tissue (McNeil, 1988). Thus faecal estimates of digestibility may be misleading and digestibility may be better determined at the terminal ileum (Sauer and Ozimek, 1986).

The aim of the present study was to determine the profile of absorbed amino acids for mature human milk. The study was conducted in two parts. The first part involved the collection of representative human milk samples and determination of the amino acid composition of each sample. Corrections were made for the losses of amino

acids that occur during hydrolysis in acid prior to amino acid analysis, using the non-linear mathematical model described in chapter 5. In part two of the study the three-week-old piglet was used as a model animal for the human infant, to determine true (corrected for endogenous amino acids) ileal digestibility coefficients for the amino acids in mature human milk. The true ileal digestible amino acid composition of human milk is compared with the recommended amino acid requirements of the infant (FAO/WHO/UNU, 1985).

## Part I: DETERMINATION OF THE AMINO ACID COMPOSITION OF MATURE HUMAN MILK

### 6.3 EXPERIMENTAL

All aspects of the human research reported in this part of the study were approved by the Massey University Human Ethics Committee.

#### 6.3.1 Collection of milk samples

Twenty women (average age (± SE), 29.6 ± 0.83) years old; mean body weight (± SE) 64.7 ± 2.08 kg; 16 were primiparous) whose pregnancies were uncomplicated, resulting in the vaginal delivery of full-term infants and who were currently nursing healthy exclusively breast-fed infants, participated in the study. At the beginning of the collection period the women were in their 10th to 14th weeks of lactation (average (± SE) 12 ± 0.3 weeks). The women were fully informed of the collection procedure.

The women participating in the study expressed samples of milk under supervision. The breast, from which milk had not been removed for at least 3-4 hours, and nipple were washed with distilled water and thoroughly dried with a sterile cotton towel. The maximum amount of milk that could be comfortably expressed (mean volume 83.9 (SE 6.01) ml) from one breast was collected using an electric breast-pump (Egnell Inc., Cary, IL). If necessary, to assist with milk let-down, the women were encouraged to suckle their infant at the contralateral breast during collection. The milk was collected into sterile, acid-washed polypropylene containers. The milk samples were gently mixed and a sub-sample (25-30 ml) taken, which was subsequently freeze-dried. The remaining milk which was to be used in the second part of this study, was stored at -20°C. The process was repeated for each woman on 10 occasions over a 20 day period. To ensure a representative sample was obtained, the women were advised

to express samples at different times during the day (ie. 3 morning, 4 afternoon and 3 evening samples) and to alternate the breast from which the milk was expressed. The 10 freeze-dried sub-samples collected from each woman were pooled together to give one representative sample per woman.

### 6.3.2 **Chemical Analysis**

The samples of freeze-dried human milk were analysed in duplicate for dry matter (DM), ash and total nitrogen (N). The DM content of the samples was determined by drying samples in a forced-air oven at 60°C until a constant weight was achieved. The samples were subsequently ashed in a furnace at 500°C for 16 hours. Total N was determined by the Kjeldahl method (AOAC, 1980).

Prior to amino acid analysis the freeze-dried milk samples were defatted with light petroleum ether (b.p. 40°C-60°C) using a Soxhlet apparatus (AOAC, 1980). The petroleum ether extractable fat percentage of the milk was determined in duplicate.

Six replicates (5-7 mg/replicate) of defatted freeze-dried human milk collected from each of the 20 women, were accurately weighed into acid-washed pyrolysed (650°C, overnight) glass test tubes (10 ml) and 1 ml of glass-distilled 6 M-HCl (containing 1% phenol w/v) was added to each tube. Four of the replicates were sealed under vacuum and hydrolysed in duplicate at 110 ± 2°C, two for 10 hours and two for 24 hours. An internal standard (10 µl of 40 mM norleucine: Sigma, St Louis, MO, USA Code: n-8513) was added to each of these hydrolysates and the 2 remaining replicates that had not been heated. The tubes were immediately dried down under vacuum (Automatic Speedvac Concentrator AS290, Savant Instruments Inc, Farmingdale, NY) and 1 ml of loading buffer (0.2 M-sodium citrate, pH 2.2) was added to each tube. The 2 unheated replicates were deproteinised non-chemically by ultrafiltration (exclusion limit: 10,000 daltons, Amicon Micropartition System, Amicon Division, Grace and Co, Danver, MA, USA). The ultrafiltrates and hydrolysates were loaded onto a Pharmacia LKB - Alpha Plus Amino Acid analyser (Cambridge, England). The amino acids were separated by ion-exchange chromatography and detected following reaction with ninhydrin. A further 6 replicates of freeze-dried human milk from each woman were also analysed for cysteine and methionine after performic acid oxidation (Moore, 1963) following the same hydrolysis procedure as for the acid-hydrolysed samples. Cysteine and methionine were detected as cysteic acid and methionine sulphone, respectively, after loading on to an ion-exchange HPLC

system (Millipore Corporation, Waters, Chromatography Division, Milford, MA, USA), with post-column *O*-phthaladehyde derivitisation and fluorescence detection. Tryptophan, being destroyed during acid hydrolysis was not determined. The free molecular weight pertaining to each individual amino acid was used to convert from moles to a gram weight.

### 6.3.4 **Data Analysis**

The amino acid compositions of the hydrolysates were corrected for losses of amino acids that occurred during acid hydrolysis using equation 1 which is based on the rearrangement of the non-linear model derived in chapter 5 that describes the simultaneous processes of amino acid yield and decay occuring during acid hydrolysis of a protein sample.

$$\mathbf{Ao} = \frac{[B(t) - \mathbf{Bo}(e^{-lt})]\ (h - l)}{h(e^{-lt} - e^{-ht})} \qquad (1)$$

Where,

**Ao** = the original amount of amino acid in protein form prior to hydrolysis,

B(t) = amount of amino acid measured at time t,

$h$ = the rate at which an amino acid is liberated from its protein-bound state during acid hydrolysis, expressed as a proportion of the amount of amino acid remaining in protein form,

$l$ = the rate at which an amino acid in free form is degraded or altered to an undetectable form during acid hydrolysis, expressed as a proportion of the amount of amino acid in free form during acid hydrolysis,

**Bo** = the amount of free amino acid measured in a sample prior to hydrolysis.

The amount of each amino acid present in the milk is the sum of **Ao** and **Bo**. The parameters $h$ and $l$ pertaining to each amino acid were determined in chapter 5. An average estimate of **Ao**, derived for each amino acid, using the 10 and 24 hour hydrolysis values, was added to the average **Bo** value to give the amino acid composition of the human milk samples. The amino acid N content of the human milk sample was determined by summation of the N contributed by each amino acid on a molecular weight basis.

## Part II: DETERMINATION OF TRUE ILEAL AMINO ACID DIGESTIBILITY IN HUMAN MILK

### 6.4 EXPERIMENTAL

All aspects of the human and animal research reported in this part of the study were approved by the Massey University Human Ethics and Massey University Animal Ethics Committees, respectively.

#### 6.4.1 **Animals and housing**

Eighteen Landrace X Large White entire male piglets (average age 6 days; mean body weight (± SE) 2.3 ± 0.12 kg) were selected at random from a group of piglets originating from nine different litters of pigs (Pig Research Unit, Massey University). The piglets were penned individually in moulded-plastic metabolism cages and were kept in a temperature controlled room maintained at 30 ± 1°C with a constant 15 hour light : 9 hour dark cycle.

#### 6.4.2 **Diets**

The 3 diets used included a preliminary diet, human milk and a protein-free (PF) diet. The latter diet was given to the piglets to allow determination of endogenous amino acid flows at the terminal ileum. The composition of the preliminary diet has been reported previously (Darragh *et al.* 1994; refer chapter 3). The composition of the PF diet (Table 6.1) was formulated to resemble human milk with respect to its carbohydrate, fat, vitamin and mineral components.

The preliminary diet was mixed with distilled water daily (14 g DM/100 ml water) and was kept refrigerated at 4°C. The PF diet was also prepared daily (14.5 g DM/100 ml water) by mixing the non-oil fraction with warm distilled water. The oils were added and the liquid homogenized at pressures of 200 kg/cm$^2$ (stage 1) and 75 kg/cm$^2$ (stage 2). The surplus human milk from part I of the study, which had been stored at -20°C, was thawed at 4°C as required. All the liquids were warmed to 35°C before feeding and each piglet's daily allocation was given in equal amounts every 2½ hours throughout the day, from 0630 to 2130 h. The meal frequency was chosen to mimic human practice.

**Table 6.1.**

*Ingredient composition of the protein-free diet used to determine endogenous amino acid flows at the terminal ileum of the three-week-old piglet*

| Ingredient | g/100 g of air-dry matter |
|---|---|
| Purified Lactose | 57.5 |
| Glucose | 8.7 |
| Palm olein* | 15.0 |
| Coconut oil* | 9.0 |
| Soya oil* | 3.0 |
| Maize oil* | 3.0 |
| Vitamins/minerals+ | 2.8 |
| Emulsifier# | 1.0 |

* The fatty acid profile was: C:8, 2.8; capric, 1.3; lauric, 15.3; myristric, 6.2; palmitic, 21.7; palmitoleic, 0.2; stearic, 5.9; arachidic, 0.07; lignoceric, 0.01; oleic, 33.9; linoleic, 12.4; and linolenic, 0.2%.

\+ The vitamin and mineral composition was: Ca, 684; P, 318; K, 697; Na, 359; Cl, 705; Mg, 44; Fe, 1.8; Zn, 2.9; Cu, 1.2; ascorbic acid, 30; vitamin E, 2.8; pantothenic acid, 2.2; and nicotinic acid, 1.8 (mg/100g); I, 85; S, 82; Se, 9.3; thiamin, 161; riboflavin, 311; pteroylmonoglutamic acid, 51; retinol, 473; pyridoxine, 47; cyanocobalamin, 80; cholecaliferol, 6.3; and biotin, 6 (µg/100g).

\# Glycerol monostearate.

### 6.4.3 **Experimental Procedure**

In the 9 days following removal from the sow and before commencement of the experimental period the piglets were trained to drink the preliminary diet from bottles with soft rubber teats attached. At each meal time the piglets were removed from their pens and individually hand-fed their diets. During each meal, the minimal spillage which occurred was collected and meal intakes were adjusted. The daily allowance for all diets was set at 42g DM/kg body weight/day. This corresponded to a level of intake on a gut capacity basis (0.923 g DM/$cm^3$ of estimated stomach volume/day) similar to that of breast-fed human infants, assuming that the piglet has a gut capacity, on a bodyweight basis, twice that of the human infant (Moughan *et al.* 1992a). The piglets were weighed (± 10g) on days 4 and 8 of the preliminary period, and their feed

intakes were adjusted.

At the end of the 9 day preliminary period each piglet was fitted with a karaya base plate and human ostomy bag around the anal region (Darragh *et al.* 1994; refer chapter 3) to allow the collection of faeces, thus preventing coprophagy.

On day 1 of the 6 day experimental period the piglets were randomly allocated to either the human milk or PF diet (6 piglets/diet) and were given a 50:50 mix of the preliminary diet and their allocated experimental diet to allow them to adapt to any variation in taste. On day 2 the piglets were weighed, the level of food intake was adjusted, and from days 2-6 of the experimental period the piglets received their allocated experimental diet. In the final 2 days of the experimental period fresh human milk was collected from a further 10 mothers in their 10th to 14th weeks of lactation, using the selection criteria and collection procedures described in Part I. The fresh human milk was pooled and stored at 1°C. On the penultimate and final day of the experimental period (days 5 and 6) the piglets allocated the human milk were given fresh human milk. It was important that the human milk fed to the piglets on the final day of the experimental period was fresh as some of the functional properties of the human milk may have been altered by freezing and storage (Garza *et al.* 1982). During the experimental period chromic oxide ($Cr_2O_3$) was added as an indigestible marker (to give a final concentration of 0.3g/100 g DM), to each piglet's daily allocation of human milk or PF diet. Before and during each feeding the diets were thoroughly mixed to ensure that an even distribution of the marker in the diet was maintained. On days 4 and 5 of the experimental period the meal frequency was changed to hourly feedings between 0630h and 2130h. It was assumed that an hourly feeding regimen would ensure a relatively constant flow of representative digesta at the end of the ileum.

On the final day of the experimental period (day 6) and beginning 10 hours after the first feeding of the day, piglets were chosen at random to be killed for the sampling of ileal digesta. Thirty minutes after a meal, each piglet was anaesthetised with halothane gas (Fluothane, Imperial Chemical Industries Ltd, Cheshire, England) and the abdominal cavity was opened. The 40 cm of ileum directly anterior to the ileo-caecal valve was isolated and dissected from the body. After removal of the ileum the piglet was killed by intracardial injection of 2 ml of sodium pentobarbitone (Anathal, 300 mg/ml). The external surface of the dissected ileal segment was rinsed with distilled water to remove residual blood and tissue. The contents of the section were flushed with distilled water from a plastic syringe and the digesta samples were

immediately frozen. The ileal digesta samples and subsamples of the fresh human milk and PF diet, were freeze-dried, finely ground and stored at -20°C while awaiting chemical analysis.

### 6.4.4 **Chemical Analysis**

The samples of freeze-dried human milk, PF diet, and the ileal digesta samples were analysed in duplicate for DM, ash and total N as described in part I. The chromium content of the diets and ileal samples were also determined in duplicate by the method of Costigan and Ellis (1987).

The amino acid composition of duplicate freeze-dried defatted samples of the human milk and the ileal samples were determined (refer Part I) following 24 hour acid hydrolysis. The determination of cysteic acid and methionine sulphone in the ileal digesta samples was not possible due to limited sample size. Free molecular weights of the amino acids were used to convert from moles to a gram weight.

### 6.4.5 **Data Analysis**

The ileal flows of N and amino acids (AA) related to the ingestion of 1g of dietary DM were calculated using equation 2 (units are $\mu g\ g^{-1}$ DM).

**N or AA Flow**

= N or AA concentration in ileal digesta x (diet chromium/ileal chromium) (2)

The apparent and true ileal digestibilities of N, amino acid N and the individual amino acids in the human milk were calculated using equations 3 and 4, respectively (all units are $\mu g\ g^{-1}$ DM).

**Apparent AA Digestibility (%)**

= [(AA Intake - Ileal AA flow)/AA intake] X 100 (3)

**True AA Digestibility (%)**

= [(AA intake -{Ileal AA flow - endogenous AA flow})/AA intake] X 100 (4)

The amino acid compositions of ileal digesta samples from the piglets fed either human milk or the PF diet were not corrected for amino acid losses occurring during hydrolysis because the parameters *h* and *l* (refer equation 1) have not been determined

for ileal digesta. Consequently, the digestibility values were calculated using the uncorrected amino acid composition determined after 24 hour hydrolysis of the pooled sample of the human milk and the ileal digesta samples.

The apparent and true digestibility data for N, amino acid N and the individual amino acids were analysed statistically using a one-way analysis of variance (GLM procedure, SAS Institute Inc, USA).

## 6.5 RESULTS

For the DM, ash, N, fat and chromium determinations, the overall mean differences between duplicates within samples (expressed as a percentage of the mean), were 0.6%, 1.83%, 0.74%, 3.77% and 6.42%, respectively. For the analysis of amino acids the overall mean differences between duplicates (expressed as a percentage of the mean) were 5.4% and 2.3% for the ninhydrin detected amino acids and sulphur amino acids, respectively.

Part I: *Determination of the amino acid composition of mature human milk*

The chemical composition of the human milk is given in Table 6.2. The non-protein N content of the human milk determined by difference was 25.6% of the total N. The crude protein content of the human milk, calculated by multiplying the total N content by the conversion factor of 6.38, was 1.13 g/100ml. Multiplying the amino acid N by 6.38 gave a protein content of 0.84 g/100ml, whereas the protein content of the milk derived by summation of the amino acids (accounting for the water of hydrolysis but excluding tryptophan) was 0.88 g/100 ml. The amino acid composition of the human milk, corrected for losses of amino acids occurring during acid hydrolysis, is given in Table 6.3.

Part II: *Determination of the true ileal digestibility of amino acids in human milk*

One piglet (PF diet) developed diarrhoea and was removed from the study. The remaining piglets appeared healthy and consumed the diets readily. The mean growth rates (± SE) during the experimental period were 72 ± 5.0 and 44 ± 8.9 g/day for the piglets fed human milk and the PF diet, respectively.

The endogenous flows of amino acids at the terminal ileum for piglets fed the PF diet (Table 6.4) were used to determine true ileal amino acid digestibility values (Table 6.5). Apparent amino acid digestibility values are also given in Table 6.5.

**Table 6.2.**

*The chemical* composition of mature human milk+*

(Mean values with their standard errors for twenty women)

| | g/100ml# | | | g/100g dry matter | |
|---|---|---|---|---|---|
| | mean | SE | range | mean | SE |
| Total Nitrogen | 0.18 | 0.003 | 0.15 - 0.20 | 1.37 | 0.026 |
| Amino Acid Nitrogen§ | 0.13 | 0.002 | 0.11 - 0.15 | 1.02 | 0.018 |
| Protein‡ | 0.88 | 0.017 | 0.74 - 0.99 | 6.85 | 0.123 |
| Fat¶ | 3.94 | 0.169 | 2.73 - 5.51 | 30.49 | 1.017 |
| Ash | 0.20 | 0.003 | 0.18 - 0.23 | 1.55 | 0.031 |

* Excluding the carbohydrate fraction which was not determined.

\+ Mean values for samples collected from 20 women (10 samples per woman), in their 10th-14th weeks of lactation.

\# 100 ml of mature human milk contained (mean ± SE) 12.9% ± 0.15% of dry matter.

§ Calculated based on summation of the nitrogen contributed by each amino acid on a molecular weight basis.

‡ Calculated based on summation of the total amino acids determined after conversion of moles to grams using the residue molecular weight.

¶ Soxhlet extractable fat only.

There were significant differences between apparent and true digestibility for total N, amino acid N and for all of the individual amino acids except valine in human milk. The lower digestibility of total N compared to the digestibility of the amino acid N in the human milk was most likely due to the lower digestibility of the non-amino acid N fraction in human milk. The true digestibility of the non-amino acid N, calculated by difference, was 50%.

The very low apparent digestibility for glycine in the human milk is indicative of a large excretion of endogenous glycine. The subsequent correction of this value for the endogenous flow of glycine resulted in a true digestibility more in line with the true digestibilities of the other amino acids. The essential amino acid threonine also had a low apparent digestibility, and remained the second least digestible amino acid in human milk after correction for endogenous amino acid excretion.

**Table 6.3.**

*The amino acid composition* of mature human milk+*

(Mean values with their standard errors for twenty women)

| Amino Acid | mg/100 ml | | | mg/g Dry Matter | | mg/g Total Nitrogen | |
|---|---|---|---|---|---|---|---|
| | mean | SE | range | mean | SE | mean | SE |
| Aspartic Acid | 102 | 2.4 | 85 - 121 | 7.9 | 0.16 | 580 | 9.3 |
| Threonine | 52 | 1.6 | 42 - 73 | 4.0 | 0.12 | 294 | 8.5 |
| Serine | 48 | 1.1 | 40 - 59 | 3.7 | 0.07 | 270 | 4.6 |
| Glutamic Acid | 189 | 3.2 | 157 - 210 | 14.6 | 0.27 | 1074 | 11.6 |
| Proline | 95 | 2.3 | 77 - 115 | 7.3 | 0.18 | 537 | 10.9 |
| Glycine | 24 | 0.6 | 19 - 29 | 1.8 | 0.04 | 135 | 1.9 |
| Alanine | 38 | 0.9 | 31 - 46 | 2.9 | 0.07 | 214 | 2.3 |
| Valine | 58 | 1.4 | 47 - 65 | 4.5 | 0.10 | 327 | 5.3 |
| Isoleucine | 57 | 1.0 | 48 - 65 | 4.4 | 0.09 | 323 | 4.3 |
| Leucine | 104 | 1.8 | 88 - 114 | 8.1 | 0.15 | 593 | 5.4 |
| Tyrosine | 42 | 0.9 | 36 - 49 | 3.3 | 0.07 | 240 | 2.0 |
| Phenylalanine | 43 | 1.9 | 32 - 72 | 3.3 | 0.14 | 242 | 9.1 |
| Histidine | 26 | 0.6 | 22 - 31 | 2.0 | 0.04 | 149 | 2.4 |
| Lysine | 70 | 1.5 | 70 - 81 | 5.4 | 0.11 | 398 | 3.8 |
| Arginine | 35 | 1.2 | 27 - 46 | 2.7 | 0.08 | 200 | 3.7 |
| Cysteine# | 29 | 0.8 | 24 - 35 | 2.3 | 0.06 | 165 | 3.0 |
| Methionine§ | 16 | 0.4 | 13 - 20 | 1.2 | 0.03 | 90 | 1.2 |
| Taurine | 7 | 0.2 | 6 - 9 | 0.6 | 0.01 | 41 | 0.9 |

* All amino acids were corrected for losses that occurred during acid hydrolysis, and were converted from moles to mg using the appropriate molecular weight including the molecular weight of the water of hydrolysis.

\+ Mean values of samples taken from 20 women (10 samples per woman), in their 10th-14th weeks of lactation.

\# Detected as cysteic acid.

§ Detected as methionine sulphone.

**Table 6.4.**

*The mean endogenous flows of total nitrogen (mg/g dry matter intake (DMI)), amino acid nitrogen (mg/g DMI) and individual amino acids (μg/g DMI) at the terminal ileum of three-week-old piglets fed a protein-free diet*

(Mean values with their standard errors for five piglets)

| | Mean Flow | SE |
|---|---|---|
| Total Nitrogen | 1.61 | 0.250 |
| Amino Acid Nitrogen | 1.03 | 0.139 |
| Aspartic Acid | 662 | 80.8 |
| Threonine | 651 | 85.2 |
| Serine | 545 | 77.4 |
| Glutamic Acid | 682 | 108.5 |
| Proline | 417 | 56.8 |
| Glycine | 1203 | 231.2 |
| Alanine | 434 | 54.5 |
| Valine | 427 | 59.7 |
| Methionine | 140 | 23.4 |
| Isoleucine | 412 | 67.7 |
| Leucine | 650 | 106.3 |
| Tyrosine | 230 | 41.1 |
| Phenylalanine | 307 | 50.3 |
| Histidine | 205 | 36.3 |
| Lysine | 244 | 64.6 |
| Arginine | 281 | 44.4 |

**Table 6.5.**

*Mean apparent and true ileal digestibility (%)[+] of the nitrogen and amino acids in mature human milk[#] given to piglets*

(Mean values and the overall standard error for six piglets)

| Amino Acid | Digestibility | | Overall SE | Level of Significance[¶] |
|---|---|---|---|---|
| | Apparent | True[§] | | |
| Total Nitrogen | 74 | 88 | 4.5 | * |
| Amino Acid Nitrogen | 84 | 95 | 2.8 | ** |
| Aspartic Acid | 86 | 95 | 2.1 | *** |
| Threonine | 65 | 86 | 5.5 | *** |
| Serine | 78 | 95 | 3.4 | *** |
| Glutamic Acid | 93 | 98 | 1.2 | *** |
| Proline | 86 | 92 | 2.7 | ** |
| Glycine | 16 | 81 | 12.9 | ** |
| Alanine | 81 | 95 | 3.5 | *** |
| Valine | 83 | 90 | 3.6 | NS |
| Methionine | 91 | 100 | 1.7 | ** |
| Isoleucine | 90 | 98 | 1.7 | ** |
| Leucine | 92 | 99 | 1.4 | *** |
| Tyrosine | 92 | 100 | 1.7 | *** |
| Phenylalanine | 82 | 93 | 3.2 | *** |
| Histidine | 86 | 95 | 2.9 | * |
| Lysine | 93 | 98 | 1.2 | ** |
| Arginine | 89 | 101 | 2.5 | *** |

[+] Digestibility values were calculated from the dietary ratio of nutrient to chromium, relative to the corresponding ratio in the ileal digesta.

[#] Pooled sample of fresh human milk from 10 women in their 10th-14th weeks of lactation.

[§] Corrected using endogenous amino acid flows determined after feeding piglets a protein free diet.

[¶] NS=non-significant, P>0.05; *=P<0.05; **=P<0.01; ***=P<0.001.

## 6.6 DISCUSSION

In the present study representative samples of mature human milk (>12 weeks lactation) were collected from women, following recommendations for the collection and storage of human milk outlined in the literature (DHSS, 1981; Hamosh *et al.* 1984). External factors such as the maternal diet (Lönnerdal, 1986), circadian variation (Hytten, 1954) and the health of the mother (Butte *et al.* 1987) will affect the composition of human milk. In general, however, the protein content does not vary significantly compared to the fat, vitamin and mineral contents of human milk. This was reflected, in the present study, in the variability of the different components of human milk (Table 6.3) with coefficients of variation (CV) of 19.2% and 8.2% for the fat and protein contents of the milk, respectively.

The average protein content (0.88 g/100 ml) of the human milk in the present study was similar to literature estimates (Lönnerdal *et al.* 1976; Hambraeus *et al.* 1978), though somewhat lower than the value of 1.07 g protein/100ml reported by the DHSS (1977). The non-amino acid N fraction (25.5%) of the total N in the human milk was also similar to previously reported estimates (Lönnerdal *et al.* 1976; DHSS, 1977; Hambraeus *et al.* 1978).

Although precise comparison is difficult, due to the variety of collection, analysis and reporting techniques used in different studies, the average gross amino acid composition of the human milk in this study was generally similar to the average of several literature estimates (DHSS, 1977; Svanberg *et al.* 1977; Casey and Hambidge, 1981; Committee on Nutrition, 1985). The concentration of cysteine, however, was some 20% higher than the average literature estimate and 16% higher than the highest previously reported estimate (DHSS, 1977). The higher cysteine value in the present study is a result, at least in part, of the correction for losses of cysteic acid when the human milk was hydrolysed in acid prior to amino acid analysis. The cysteic acid in human milk has been shown in earlier studies (refer chapters 4 and 5) to experience a loss rate during acid hydrolysis greater than that shown by serine. Although cysteine is not generally recognised as an essential amino acid in the human adult, there is some suggestion that cysteine is essential for the human infant (Sturman *et al.* 1970), signifying that accurate determination of the cysteine content of human milk is necessary. To this end, correction for losses of cysteic acid during acid hydrolysis should be made.

The gross amino acid composition of human milk is used as a standard for

determining the amino acid requirements of the infant, assuming that the amino acids in human milk are completely absorbed by the infant (FAO/WHO/UNU, 1985). It has been suggested, however, that several of the proteins in human milk resist digestion (Hambraeus *et al.* 1984), as significant amounts of intact immune proteins have been found in the faeces of breast-fed infants. Van Woelderen (1987) proposed that the digestible protein in human milk could be determined by making a correction for the faecal immune proteins. The faecal method for determining amino acid digestibility, however, leads to considerable overestimation of digestible protein, due to the significant microbial breakdown of protein, mainly to ammonia, that occurs in the large intestine (McNeil, 1988).

An alternative approach is to determine the digestibility of amino acids in human milk at the end of the small intestine taking into consideration the endogenous excretion of amino acids. The routine collection of ileal digesta from human infants is not practical, however, thus dictating the use of a model animal.

The piglet would appear to be a suitable model animal for studying aspects of protein digestion in human infants. A comparative review (Moughan *et al.* 1992a) of the digestion of protein in milk-fed piglets and human infants highlights anatomical and physiological similarities in the digestive processes of these species, especially when comparison is made at the physiologically comparable age corresponding to peak milk output in the mother (approximately 3 weeks in the piglet and 3 months in the human infant). Furthermore, direct comparison of the digestion of a high quality protein in human infants and piglets (refer chapter 2) has demonstrated that the two species have a similar capacity to digest protein, although the comparison was limited to faecal measurements. Validation of the piglet as a model for the infant at the ileal level is more difficult. Direct comparison of the digestion of proteins to the end of the small intestine in adult humans and growing pigs, however, has shown similarities between the two species in their capacity to digest protein (Rowan *et al.* 1994). In the present study, therefore, the three-week-old piglet was accepted as a suitable model for the human infant to determine the true ileal amino acid digestibility of human milk, with experimental conditions being chosen to closely mimic the feeding of milk to human infants.

The ileal amino acid flows in piglets given human milk were corrected for endogenous amino acid flows, to give true ileal digestibility values. Endogenous ileal amino acids flows can be determined after feeding an animal a protein-free (PF) diet.

The PF method has been criticised, however, as being 'unphysiological', and endogenous flows may be underestimated (Low, 1980). In the present study, the endogenous amino acid flows determined using the PF method were, in general, greater than those previously determined in 15-20 kg pigs (Moughan *et al.* 1992b; Butts *et al.* 1993) with the exception of glutamic acid which was lower and proline which was up to 8 fold lower when compared to previous estimates. Proline appears to be the most abundant amino acid in the endogenous excretion collected at the terminal ileum of pigs fed PF diets (Moughan and Schuttert, 1991; Moughan *et al.* 1992b; Butts *et al.* 1993). The abundance of proline in the digesta of pigs receiving a PF diet may be directly related to the protein status of the pig. In animals deprived of dietary protein, body protein, primarily in the form of muscle, is broken down to supply amino acids essential for metabolism. Alanine and glutamine account for more than 50% of the total α-amino acid N released from muscle tissue (Rodwell, 1985). The intestinal tissue takes up large quantities of glutamine which in turn can be metabolised to proline (Rodwell, 1985), which it has been suggested (Sauer and de Lange, 1992), leads to an increase in the excretion of both glutamine and proline into the gut lumen. When determining the endogenous excretion of amino acids using the PF method, the standard procedure is to feed the PF diet to the animal for 7 or more days prior to collection of digesta. In this study the piglets were fed the PF diet exclusively for only 4 days. Given the low excretion of proline and glutamic acid compared with other studies, it is possible that the piglets did not receive the protein-free diet long enough to induce major muscle breakdown. An alternative and possibly more physiological approach to determining endogenous ileal amino acid flows, whereby ileal samples are collected from animals fed an enzymically hydrolysed protein which is then processed to remove undigested dietary N (Butts *et al.* 1993), was used here in a preliminary study (AJ Darragh, unpublished) to determine endogenous ileal amino acid flows in the three-week old milk-fed piglet. There was concern, however, about the validity of these data, as difficulties were encountered in obtaining representative digesta samples. In this case, the PF-derived endogenous amino acid flows, determined in the present study, were used to correct the ileal amino acid flows in the piglets fed human milk.

The true ileal digestibilities of the amino acids in human milk ranged from 81%-101%. The true ileal digestibility of glycine (81%) needs to be interpreted with caution, however, as an overestimation of the endogenous flow of glycine in PF-fed animals has been reported (Moughan *et al.* 1992b). The apparent glycine digestibility value of 16%

suggests that the endogenous excretion of glycine was considerable in the piglets fed human milk. If the endogenous glycine flow is an overestimation, the true ileal digestibility of glycine may be even lower than reported here. Jackson (1989) suggested that human milk may be an inadequate source of glycine for the infant, based on the small amount of glycine present in human milk. The results from the present study suggest that the digestible glycine content of human milk is even lower.

In addition to glycine, the amino acids threonine, proline and valine were also less digestible compared to the other amino acids in human milk. These amino acids are present in greater proportions in the immune proteins lactoferrin, secretory IgA and lysozyme, compared to the other proteins in human milk (Harzer and Bindels, 1987). Their lower true digestibility may be indicative of a reduced digestibility of the immune proteins in the piglet's intestine. This has direct implications for the determination of amino acid requirements for human infants with the digestible rather than gross amino acid composition of human milk being a more appropriate base. Further, given the differences in the digestibility of individual amino acids, the digestible amino acid profile of human milk should be determined using individual amino acid digestibility values rather than a single N digestibility value.

The amino acid composition of human milk and the digestibility values determined in the present study were used to derive a profile of digestible amino acids for milk. The pattern of digestible amino acids (relative to lysine) was somewhat different from the pattern reported by the FAO/WHO/UNU (1985) as representative of the amino acid requirements for infants (Table 6.6). A combination of correction for hydrolytic losses of amino acid during acid hydrolysis and for amino acid digestibility resulted in a significant change in the proportions of the essential amino acids relative to lysine. When the digestible rather than gross amino acid composition of human milk was used as a standard, the total amount of essential amino acids relative to lysine increased. The proportions of isoleucine, leucine and the aromatic amino acids increased, while the proportions of histidine and to a greater extent valine decreased relative to lysine in the pattern of digestible amino acids. Higher concentrations of valine have been reported in the plasma of formula-fed infants compared to breast-fed infants (Räihä *et al.* 1986; Lönnerdal and Chen, 1990). Although, this may in part be due to the higher intake of valine in formula-fed infants, the extent of the difference may be accentuated by the lower intake of digestible valine relative to lysine in breast-fed infants.

**Table 6.6.**

*The profile* of digestible essential+ amino acids in mature human milk compared with the profile of amino acids deemed to be required by the human infant*

| Amino Acid | Digestible Pattern# | Recommended Pattern§ |
|---|---|---|
| Lysine | 100 | 100 |
| Histidine | 37 | 39 |
| Isoleucine | 79 | 70 |
| Leucine | 148 | 141 |
| Methionine/Cysteine | 65¶ | 64 |
| Phenylalanine/Tyrosine | 122 | 109 |
| Threonine | 65 | 65 |
| Valine | 75 | 83 |

* Expressed relative to lysine = 100 units.
\+ Excluding tryptophan.
\# Corrected based on the true ileal amino acid digestibility of human milk.
§ FAO/WHO/UNU (1985).
¶ Assuming that the true ileal digestibility of cysteine was 100%.

The overall true ileal digestibility of the protein (95%) in human milk in the present study was higher than expected (Hambraeus *et al.* 1984; Davidson and Lönnerdal, 1987). The piglet may have digested the human milk more efficiently than would be expected in the breast-fed infant, however, with the immune proteins in the human milk possibly being more susceptible to digestion in the piglet. Lactoferrin can exist in either an iron-saturated form or free of iron, with the iron-saturated form being more resistant to digestion (Brock *et al.* 1976). Although lactoferrin in human milk is only saturated to a very limited extent (Fransson and Lönnerdal, 1980), it has been suggested that iron present in other compartments in the milk may be released and transferred to lactoferrin (Lönnerdal, 1985), thus increasing lactoferrin's resistance to digestion. There is the possibility that saturation of the lactoferrin did not occur in the piglet's intestine, rendering lactoferrin more susceptible to digestion. Schmitz *et al.* (1988) demonstrated, however, that a significant proportion of bovine lactoferrin fed to three-week-old piglets was detected at the end of the ileum suggesting that proteolytic resistance of the immune proteins may not be species-specific.

It is concluded that the absorbed rather than gross amino acid composition of

human milk is a more appropriate base from which to determine the amino acid requirements of infants and that when the absorbed amino acid profile of human milk is determined, the pattern of amino acid requirements is different compared to the recommended pattern. Thus, the current profile of amino acids deemed to be required by the human infant (FAO/WHO/UNU, 1985), which is also used to evaluate protein quality in foods for infants under 1 year of age, needs to be reassessed. For absolute accuracy in determining the digestible amino acid composition of human milk, corrections should be made for losses of amino acids which occur when human milk is hydrolysed in acid prior to amino acid analysis. True ileal amino acid digestibility values should be used to correct the gross amino acid composition of human milk to account for differences in digestibility among the amino acids.

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# CHAPTER 7

# ILEAL AMINO ACID DIGESTIBILITY IN AN INFANT FORMULA DETERMINED USING THE THREE-WEEK-OLD PIGLET AS A MODEL ANIMAL FOR THE HUMAN INFANT

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In a practical application of the information obtained in the present work, the profile of digestible amino acids for an infant formula was compared with the profile of digestible amino acids in human milk (chapter 6). The aim of the comparison described in the following chapter was to evaluate the protein quality of the infant formula.

## 7.1 ABSTRACT

The piglet was used as a model animal for the human infant to determine true (corrected for endogenous amino acid excretion) ileal amino acid digestibility in a bovine-milk-based infant formula. Three-week-old male piglets (n=6) were fed the infant formula in a manner that mimicked human infant feeding practice, for a 9 day preliminary period followed by a 6 day experimental period. Chromic oxide was added to the diet as an indigestible marker (0.3g $Cr_2O_3$/100g dry matter). At the end of the experimental period the piglets were anaesthetised and samples of digesta were removed from the terminal 40cm of each piglet's ileum. Endogenous ileal amino acid flows in three-week-old piglets fed a protein-free diet, were used to calculate true ileal amino acid digestibility coefficients for the infant formula. There was a significant ($P<0.01$) difference between apparent and true ileal digestibility values, with the latter values ranging from 95% for lysine to 103% for arginine, indicating near-complete digestion of the protein in the infant formula. The lower digestibility of lysine may indicate a degree of heat damage. The profile of absorbed amino acids for the infant formula is compared with a profile of absorbed amino acids for human milk.

## 7.2 INTRODUCTION

The currently recommended amino acid requirements for the healthy full-term infant are based on the amino acid composition of human milk (FAO/WHO/UNU, 1985), and the amounts of amino acids consumed by the breast-fed infant are considered as a minimum when establishing standards for the manufacture of infant formulas.

In practice, infant formulas contain more protein than human milk which is to account for a supposedly lower quality of the constituent protein. The effect of differing levels of protein intake on an infant's nitrogen (N) metabolism has been extensively researched (Young and Pelletier, 1989), and a reduction in the protein content of formulas is recommended in face of elevated urea and amino acid concentrations in the plasma of formula-fed infants compared to breast-fed infants. When the level of protein in an infant formula is reduced, however, the safety margin between the amount of balanced amino acids supplied and that required, decreases. It is imperative, therefore, that the adequacy of the dietary protein in meeting the infant's requirements, is taken into account.

The currently recommended approach to evaluation of the quality of protein in an infant formula (FAO/WHO, 1990), involves scoring the amino acid content of the infant formula relative to the gross amino acid composition of human milk. A correction for the digestibility of the protein in the infant formula is made based on true faecal N digestibility values determined with the growing rat. The lowest scoring essential amino acid determines the protein's final score. This method, however, has several limitations. Firstly, it is assumed that all of the amino acids in human milk are fully absorbed. It would appear, however, based on evidence present in the literature (Hambraeus *et al.*, 1984), and the results reported in chapter 6 of this thesis, that several of the proteins in human milk are at least partially resistant to digestion. Thus, the digestible rather than gross amino acid composition of human milk would be of more nutritional importance to the infant. Secondly, faecal estimates of digestibility can be misleading and digestibility may be better determined at the terminal ileum (Moughan and Donkoh, 1991), as there is considerable microbial degradation of protein in the large intestine (McNeil, 1988) and amino acids do not appear to be absorbed in the colon (McNeil, 1988). Also, determining the digestible amino acid composition of a protein using an overall N digestibility correction factor does not allow for variation in the digestibility of individual amino acids. Finally, scoring a protein based on the

essential amino acid with the lowest concentration does not address the issue of excess amino acids, as all amino acids scoring greater than 1 are adjusted to unity.

The aim of this study was to determine true (corrected for endogenous amino acids) ileal amino acid digestibility in a milk-based formula using the three-week-old piglet as a model animal for the human infant. Using the digestible amino acid composition of human milk (refer chapter 6) as the standard, a digestibility corrected amino acid score was determined for the infant formula. The amounts of amino acids estimated to be absorbed by breast-fed and formula-fed infants were compared.

## 7.3 EXPERIMENTAL

All aspects of the animal research were approved by the Massey University Animal Ethics Committee.

### 7.3.1 **Animals and housing**

Six Landrace X Large White male piglets (average age 6 days; mean body weight ± SE, 3.0 ± 0.15kg) were selected at random from a group of piglets originating from three different litters of pigs at the Pig Research Unit, Massey University. The piglets were penned individually in moulded-plastic metabolism cages and were kept in a temperature controlled room maintained at 30 ± 1°C and with a constant 15 hour light : 9 hour dark cycle.

### 7.3.2 **Diet and feeding**

The piglets were fed a commercial infant formula, the composition of which is given in Table 7.1. The formula was mixed with distilled water (14g dry matter (DM)/100ml water) daily and was kept refrigerated at 4°C. The daily allowance was set at 336ml formula/kg bodyweight/day. This corresponded to a level of intake on a gut capacity basis (0.923g DM/$cm^3$ of estimated stomach volume/day) similar to that of a three-month-old formula-fed infant, assuming the piglet has a gut capacity, on a bodyweight basis, twice that of a human infant (Moughan *et al.*, 1992). The liquid formula was warmed to 35°C before feeding and each piglet's total daily allocation was given in equal amounts 2½ hourly throughout the day from 0630 to 2130 h. The meal frequency was designed to mimic human practice.

**Table 7.1.**

*Ingredient and tabulated nutrient composition[+] of a commercial bovine-milk-based infant formula*

| Ingredient | g/100ml[*] |
|---|---|
| Skim Milk Powder | 2.08 |
| Demineralised Whey Powder | 3.47 |
| Lactose | 3.30 |
| Palm Oil | 1.16 |
| Coconut Oil | 1.16 |
| Soybean Oil | 1.16 |
| Minerals/Vitamins[#] | 0.22 |
| **Nutrient** | **g/100ml[*]** |
| Crude Protein[§] | 1.53 |
| Fat | 3.59 |
| Carbohydrate (lactose) | 7.17 |
| Ash | 0.27 |
| Energy (MJ GE/100ml) | 27.32 |
| Essential Amino Acids (mg/100ml) | |
| Lysine | 118.38 |
| Histidine | 39.39 |
| Methionine | 49.60 |
| Threonine | 74.61 |
| Tryptophan | 22.96 |
| Isoleucine | 90.60 |
| Leucine | 157.06 |
| Phenylalanine | 66.50 |
| Valine | 92.24 |

[+] The amino acid contents were determined except for that of tryptophan; all other values are taken from the manufacturer's specifications.

[*] The formula was mixed with distilled water to contain 12.54g dry matter/100ml formula.

[#] Minerals and vitamins added to supply: calcium, 44.52; phosphorus, 29.72; potassium, 59.45; sodium, 15.92; chloride, 40.38; magnesium, 4.81; iron, 1.05; zinc, 0.53; vitamin E, 0.99; ascorbic acid, 5.87; niacin, 1.05; pantothenic acid, 0.23 and choline, 10.61 (mg/100ml formula); manganese, 16.30; copper, 49.54; iodine, 6.39; vitamin K, 5.65; thiamin, 70.74; riboflavin, 106.10; vitamin $B_6$, 44.52; vitamin $B_{12}$, 0.13; retinol, 0.64; cholecalciferol, 1.05; folic acid, 5.30 and biotin, 1.55 (μg/100ml formula).

[§] 60% Whey protein:40% Casein protein.

### 7.3.3 **Experimental Procedure**

In the 9 days following removal from the sow and before commencement of the 6 day experimental period the piglets were trained to drink the infant formula from bottles with soft rubber teats attached. At each meal time the piglets were removed from their pens and individually hand-fed their diets. During each meal any spillages were collected and the meal intakes were adjusted accordingly. The piglets were weighed (± 10g) on days 4 and 8 of the preliminary period, and their feed intakes were adjusted.

At the end of the 9 day preliminary period each piglet was fitted with a karaya base plate and human ostomy bag around the anal region (Darragh *et al.*, 1994; refer chapter 3) to allow the collection of faeces, thus preventing the practice of coprophagy. During the experimental period chromic oxide ($Cr_2O_3$) was added as an indigestible marker (to give a final concentration of 0.3g $Cr_2O_3$/100g DM), to each piglet's daily allocation of infant formula. Before and during each feeding the formula was thoroughly mixed to ensure that an even distribution of the marker in the liquid was maintained. On days 4-6 of the 6 day experimental period the meal frequency was changed to hourly feedings between 0630 and 2130h. It was assumed that an hourly feeding regimen would ensure a relatively constant flow of representative digesta at the end of the ileum.

On the final day of the experimental period and beginning 10 hours after the first feeding of the day, piglets were chosen at random to be killed for the sampling of ileal digesta. Thirty minutes after a meal, each piglet was anaesthetised with halothane gas (Fluothane, Imperial Chemical Industries Ltd, Cheshire, England) and the abdominal cavity was opened. The 40 cm of ileum directly anterior to the ileo-caecal valve was isolated and dissected from the body. After removal of the ileum the piglet was killed by intracardial injection of 2ml of sodium pentobarbitone (Anathal, 300 mg/ml). The external surface of the dissected ileal segment was rinsed with distilled water to remove residual blood and tissue. The contents of the section were gently flushed out with distilled water from a plastic syringe and the digesta samples were immediately frozen. The ileal digesta samples and subsamples of the infant formula, were freeze-dried, finely ground and stored at -20°C while awaiting chemical analysis.

### 7.3.4 **Chemical Analyses**

Samples of freeze-dried infant formula and the ileal digesta samples were analysed in duplicate for DM, ash, total N and chromium. Dry matter was determined

by drying samples in a forced-air oven at 60°C until a constant sample weight was achieved. The samples were subsequently ashed in a furnace at 500°C for 16 hours. Total N was determined by the Kjeldahl method (AOAC, 1980). The chromium content of the formula and ileal digesta samples were also determined in duplicate by the method of Costigan and Ellis (1987).

Samples of freeze-dried infant formula, defatted with light petroleum ether (b.p. 40°-60°C) using the Soxhlet method (AOAC, 1980), and the ileal digesta samples, were analysed for amino acids. Duplicate samples (5-7 mg) were hydrolysed in 1ml of 6 M glass-distilled HCl (containing 1% phenol w/v) for 24 hours at 110° ± 2°C in glass tubes sealed under a vacuum. After hydrolysis an internal standard (10µl of 40 mM norleucine) was added to each tube. The tubes were subsequently dried under vacuum (Automatic Speedvac Concentrator AS290, Savant Instruments Inc, Farmingdale, NY) and 1ml of loading buffer (0.2 M sodium citrate, pH 2.2) was added to each sample. The samples were loaded on to a Pharmacia LKB - Alpha Plus Amino Acid Analyser (Cambridge, England), separated by ion-exchange chromatography and the amino acids detected following reaction with ninhydrin. Methionine and cysteine were also determined in the infant formula after performic acid oxidation, and detection as methionine sulphone and cysteic acid, respectively, after loading onto an ion-exchange HPLC system (Millipore Corporation, Waters, Chromatography Division, Milford, MA, USA). Tryptophan, being destroyed during acid hydrolysis, was not determined. Free molecular weights were used to convert from moles to gram weight.

### 7.3.5 **Data Analysis**

The flows of N, amino acid N (calculated by summation of the N contributed by each amino acid on a molecular weight basis) and amino acids at the terminal ileum of the piglet, related to the ingestion of 1g of DM, were calculated using equation 1 (units are µg $g^{-1}$DM).

**N or AA Flow**

= N or AA concentration in ileal digesta x (diet chromium/ileal chromium) (1)

The apparent ileal N and amino acid digestibilities were calculated using equation 2. Mean endogenous flows of N and amino acids determined in three-week-old piglets fed a protein-free diet (refer chapter 6), were used in equation 3 to calculate the true ileal digestibilities of N and the amino acids in the infant formula.

**Apparent AA Digestibility (%)**

$$= [(AA\ Intake - Ileal\ AA\ flow)/AA\ intake] \times 100 \quad (2)$$

**True AA Digestibility (%)**

$$= [(AA\ intake - \{Ileal\ AA\ flow - endogenous\ AA\ flow\})/AA\ intake] \times 100 \quad (3)$$

The apparent and true digestibility values for total N, amino acid N and the individual amino acids were compared statistically using Student's *t* test (Snedecor and Cochran, 1982).

## 7.4 RESULTS AND DISCUSSION

For DM, ash, N and chromium determinations, the overall mean differences between duplicates within samples (expressed as a percentage of the mean), were 0.6%, 1.83%, 0.74% and 6.42%, respectively. For the analysis of amino acids the overall mean differences between duplicates (expressed as a percentage of the mean) were 5.4% and 2.3% for the ninhydrin detected amino acids and sulphur amino acids, respectively. All the piglets readily consumed the infant formula and appeared healthy throughout the study. The mean (± SE) growth rate during the experimental period was 113 ± 4.3 g/day.

Sarwar *et al.* (1989) suggested that the baby pig may be better than the young rat for assessing the protein quality of infant formulas. The piglet has been used on numerous occasions as a model animal for studying aspects of protein digestion in the human infant (Newport and Henschel, 1985; Moughan *et al.*, 1990). A recent review (Moughan *et al.*, 1992) of the digestive process in milk-fed piglets and human infants highlights major anatomical and physiological similarities in the digestive process between the two species, especially when comparison is made at the physiologically comparable age corresponding to peak milk output in the mother (approximately 3 weeks in the piglet and 3 months in humans). Furthermore, direct comparison of the digestion of a high quality protein in human infants and piglets (refer chapter 2) has demonstrated a similar capacity to digest protein in the two species. Therefore, the piglet was assumed to be a suitable model animal for the human infant for determining amino acid digestibility in an infant formula and experimental conditions were chosen to closely mimic the feeding of formulas to human infants.

The determined apparent ileal digestibility values (Table 7.2) were similar to those reported in the literature for piglets given milk-based and soya-based formulas

(Moughan *et al.*, 1990). For calculation of the true digestibility values, the ileal amino acid flows of the formula-fed piglets were corrected for endogenous excretion of amino acids using values determined in piglets given a protein-free (PF) diet (refer chapter 6). The protein-deplete dietary state can lead to a reduction in the endogenous flows of amino acids with the exception of glycine, glutamic acid and proline flows which may increase (Low, 1980). Glutamic acid and proline are thought to increase as the result of muscle breakdown (Sauer and de Lange, 1992). When determining the endogenous

**Table 7.2.**

*The mean apparent and true ileal digestibility of nitrogen and amino acids in a bovine-milk-based formula fed to three-week-old piglets*

(Mean values and the overall standard error for six piglets)

| | Digestibility[+] | | | |
|---|---|---|---|---|
| | **Apparent** | **True[*]** | **Overall SE** | **Level of Significance[#]** |
| Total Nitrogen | 91 | 102 | 1.0 | *** |
| Amino Acid Nitrogen | 95 | 101 | 0.7 | *** |
| Aspartic Acid | 95 | 101 | 0.5 | *** |
| Threonine | 88 | 99 | 1.5 | *** |
| Serine | 91 | 102 | 1.3 | *** |
| Glutamic Acid | 96 | 99 | 0.5 | *** |
| Proline | 95 | 100 | 0.8 | *** |
| Alanine | 93 | 101 | 1.0 | *** |
| Valine | 95 | 100 | 0.6 | *** |
| Methionine | 97 | 101 | 0.4 | *** |
| Isoleucine | 96 | 100 | 0.5 | *** |
| Leucine | 97 | 101 | 0.4 | *** |
| Tyrosine | 97 | 102 | 0.5 | *** |
| Phenylalanine | 96 | 102 | 0.7 | *** |
| Histidine | 96 | 101 | 0.6 | *** |
| Lysine | 92 | 95 | 0.8 | ** |
| Arginine | 94 | 103 | 1.1 | *** |

[+] Digestibility values were calculated from the dietary ratio of nutrient to chromium, relative to the corresponding ratio in the ileal digesta.

[*] Corrected for endogenous amino acid flow.

[#] **=P<0.01; ***=P<0.001.

excretion of amino acids using the PF method, the standard procedure is to feed the PF diet to the animal for 7 days or more prior to the collection of digesta. In the study reported in chapter 6, however, the piglets were given the PF diet for only 4 days, which may not have been long enough to induce major muscle breakdown. As it was likely that the endogenous flow of glycine was overestimated in the piglets fed the PF diet, however, values for the apparent and true digestibility of glycine were not reported in the present study.

There were significant ($P<0.01$) differences between apparent and true ileal digestibility for total N, amino acid N and for each individual amino acid (Table 7.2), illustrating the need to correct for endogenous amino acid excretion. Most of the amino acids in the formula had a true ileal digestibility close to 100%, indicating that the amino acids in the infant formula, with the exception of lysine, were completely digested and absorbed. Lysine is particularly susceptible to structural alteration during heat-treatment of infant formulas (Sarwar *et al.*, 1988). The lower true digestibility of lysine compared to the other amino acids in infant formula, suggests that the lysine in the infant formula had undergone some degree of heat-damage.

The true ileal amino acid digestibility values determined in the present study can be used to correct the gross amino acid composition of the infant formula to provide a profile of absorbed amino acids. This profile can be compared with the profile of amino acids absorbed from human milk determined in chapter 6, to evaluate the quality of the infant formula (Table 7.3). In the present study, all of the essential amino acids scored greater than unity. Cumulatively, the sulphur amino acids had a score of 1.08. Methionine in the formula was well in excess of the methionine content of human milk, whereas cysteine in the formula was markedly deficient when compared to the cysteine content of human milk.

The most limiting essential amino acid determines the final score of the protein being evaluated (FAO/WHO, 1990). Therefore, the final score for the infant formula was 1.01 as determined by leucine, indicating a high quality protein. This raises the question, however, as to which amino acids should be classified as essential. Jackson (1989) argues that only glutamic acid, aspartic acid and alanine are truly non-essential, with the remaining amino acids being classified as either essential or conditionally essential. Although not considered essential in the adult, glycine (Jackson, 1989), histidine (Holt and Snyderman, 1965) and cysteine (Sturman *et al.*, 1970) are all thought to be conditionally essential in the infant. Using cysteine as the most limiting amino

acid would have reduced the score of the formula considerably. It must be noted, however, that in the present study, the amino acid composition of the formula had not been corrected for losses of amino acids which occur when hydrolysing a protein sample in acid prior to amino acid analysis. This is particularly relevant for the cysteine content of the infant formula, as cysteic acid exhibits a loss rate greater than

**Table 7.3.**

*Comparison of digestible amino acid profiles in human milk (HM)[+] and a bovine-milk-based infant formula (IF)[*] and anticipated daily amino acid intakes by three-month-old breast-fed or formula-fed infants.*

| Amino Acid | Amino acid Profile (mg/g total amino acid[#]) | | | Total Intake (mg amino acid/day) | |
|---|---|---|---|---|---|
| | HM | IF | Amino Acid Score[§] | HM[¶] | IF[°] |
| Aspartic Acid | 98.7 | 88.7 | 0.90 | 800 | 1198 |
| Threonine | 45.6 | 50.0 | 1.10 | 369 | 675 |
| Serine | 46.5 | 44.3 | 0.95 | 376 | 597 |
| Glutamic Acid | 188.7 | 180.0 | 0.95 | 1528 | 2429 |
| Proline | 89.0 | 75.8 | 0.85 | 721 | 1023 |
| Alanine | 36.8 | 43.7 | 1.19 | 298 | 590 |
| Valine | 53.2 | 62.4 | 1.17 | 431 | 842 |
| Isoleucine | 56.9 | 61.3 | 1.08 | 461 | 827 |
| Leucine | 104.9 | 106.3 | 1.01 | 850 | 1434 |
| Tyrosine | 42.8 | 39.9 | 0.93 | 347 | 539 |
| Phenylalanine | 40.7 | 45.0 | 1.10 | 330 | 607 |
| Histidine | 25.2 | 26.7 | 1.06 | 204 | 360 |
| Lysine | 69.9 | 76.1 | 1.09 | 566 | 1027 |
| Arginine | 35.7 | 30.2 | 0.85 | 289 | 408 |
| Methionine | 16.3 | 33.6 | 2.06 | 132 | 453 |
| Cysteine | 29.5 | 15.7 | 0.53 | 239 | 211 |

[+] Values for human milk are from Chapter 6 and have been corrected for loss of amino acids during acid hydrolysis.

[*] Calculated using true ileal amino acid digestibilities (Table 7.2) to correct the gross amino acid composition to a digestible amino acid profile.

[#] The total amino acids do not include tryptophan or taurine.

[§] Adapted from the FAO/WHO (1990) Amino Acid Scoring System: g of digestible amino acid in 100 g of test protein (infant formula)/g digestible amino acid in the reference protein (human milk).

[¶] 900ml of human milk containing 0.9g protein/100ml consumed per day.

[°] 900ml of infant formula containing 1.5g protein/100ml consumed per day.

that observed for serine during acid hydrolysis as shown in chapters 4 and 5. Accordingly, the score for cysteine would most likely have improved had a correction been made for losses of cysteic acid during acid hydrolysis.

As most infant formulas contain a higher concentration of protein compared to human milk, evaluation of an infant formula should take into consideration both the quality and quantity of the protein (FAO/WHO, 1990; Sarwar *et al.*, 1989). A method of "amino acid rating" has been developed (Sarwar *et al.*, 1989) which involves the multiplication of the amino acid score by the protein content of the formula (g/100 kcal). This value is then divided by an amino acid rating calculated for human milk. Using this technique the present formula had an amino acid rating of 1.91 (with leucine as the most limiting essential amino acid). This implies that the infant formula was providing protein well in excess of the infant's requirement for protein. This is clearly demonstrated when total daily amounts of amino acids expected to be absorbed by both breast-fed and formula-fed infants are compared (Table 7.3). Of particular note is the amount of methionine that would be consumed by the formula-fed infant relative to the breast-fed infant. The metabolic consequences of the resulting elevated concentrations in the plasma of infants are not fully understood (Young and Pelletier, 1989).

Maintaining a protein concentration in infant formulas higher than that found in human milk is only necessary if the amino acid profile is inadequate or the digestibility of the amino acids in the protein is low. The results from the present study demonstrate that the protein in the infant formula was of high quality. There would not appear to be any need, therefore, to maintain a high protein content in formulas to allow for inferior amino acid digestibility. When the adequacy of the profile of digestible amino acids is considered, reducing the protein content of the formula from 1.50 g/100ml to 0.89 g/100ml would still have provided the formula-fed infant with the required amounts of essential amino acids. If cysteine is classified as an essential amino acid for the infant, however, the protein content would need to be increased to 1.69 g/100ml to provide the same amount of cysteine consumed by the breast fed infant.

Using the three-week-old piglet as a model animal for the human infant, it was concluded that the quality of protein in the infant formula investigated was high, and that maintaining a high concentration of protein in the formula compared to that in human milk, on the grounds of inferior protein quality, is not necessary.

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# CHAPTER 8

# GENERAL SUMMARY AND DISCUSSION

Human milk appears to be uniquely adapted to the infant's physiological needs, providing the infant with both nourishment and immunological protection, and as such, breast-feeding is recommended as the exclusive form of nourishment for the infant during the first six months of life (FAO/WHO/UNU, 1985). When a mother can not or chooses not to breast-feed her infant, however, a suitable human milk substitute is necessary. Identifying the nutrient requirements of infants assists in the development of such substitutes.

Estimates of the amino acid requirements for infants have been determined based on the gross amino acid composition of human milk (FAO/WHO/UNU, 1985). This may not be appropriate, however, particularly as several proteins in human milk appear to be resistant to digestion and as such may not contribute amino acids for nutrition. The pattern of amino acids absorbed from human milk may be a better basis for determining amino acid requirements for infants.

While it is possible to obtain an indication of the absorption of amino acids from human milk by examining the plasma amino acid profiles of breast-fed infants (Lindblad *et al.*, 1978; Lucas *et al.*, 1981; Janas *et al.*, 1985), this technique is not quantitative, being better suited to relative comparisons of different dietary amino acid compositions (ie. human milk versus infant formulas). Alternatively, an assessment of amino acids absorbed from human milk can be made indirectly after determination of the digestibility of human milk protein. There is considerable microbial degradation of protein in the large intestine, however, and faecal amino acid digestibility coefficients determined in breast-fed infants are of limited value. Determining the digestibility of protein to the end of the small intestine, with correction for the endogenous excretion of amino acids, is generally considered to provide a more accurate assessment of the true digestibility of dietary protein (Sauer and Ozimek, 1986; Moughan and Donkoh, 1991). Sampling of ileal contents in human infants is difficult, however, necessitating the use of a model animal.

The piglet has been suggested as an appropriate model for studying aspects of

protein digestion in human infants (Miller and Ullrey, 1987; Moughan *et al.*, 1992). However, there has been no direct comparison of protein digestion in the human infant and piglet. Therefore, a study was conducted (chapter 2) to compare the faecal amino acid digestibility of an infant formula fed to three-month-old human infants and three-week-old piglets. Prior to discussion of the infant/piglet comparison, one result from chapter 2 that is worthy of specific comment is the finding that apparent faecal digestibility of the total nitrogen in the infant formula, determined in the human infant, was considerably higher than previously reported in the literature (see Table 8.1).

**Table 8.1.**

*Estimates of apparent faecal nitrogen digestibility in milk-formula-fed* human infants*

| Age (days) | n | Average Digestibility %+ | Experimental Technique# | Reference |
|---|---|---|---|---|
| **86** | **6** | **94.5** | **10 d collection** | **Present Study** |
| 7 | 9 | 88.0 | 3 d collection | Slater (1961) |
| 15 | 5 | 89.2 | 3 d collection | Southgate & Barrett (1966) |
| 42 | 3 | 90.2 | 8 d collection | Widdowson (1965) |
| 56-112 | 8 | 87.3 | 3 d collection | Fomon (1960) |
| 90-100 | 13 | 88.4 | 3 d collection | Fomon (1961) |
| 217 | 6 | 88.0 | 4 d collection | Cordano *et al.* (1988) |

* The milk-formulas were all bovine-milk-based.
\+ Apparent Faecal Digestibilities (%).
\# Experimental Technique: inert digestibility marker such as $Cr_2O_3$ or total faecal collection (d=day).

As mentioned in the discussion of chapter 2, it is possible that the potential for methodological error in this study increased as a result of the infant being studied at home rather than in a metabolic ward environment used in the other studies reported in Table 8.1. Every care was exercised in the conduct of the present study, however, and it is unlikely that small errors in the measurement of formula intake, or faecal output, would have resulted in such large differences between the present value and the literature estimates. It is possible, however, that the 3-4 day collection period commonly used in infant studies may be insufficient to achieve a representative faecal collection. This would seem a likely explanation for the differences observed in Table

8.1, particularly as the results obtained by Widdowson (1965), over an 8 day collection period, were closer to those found in the present study. Another possible explanation for the differences observed is the effect confinement in a metabolic ward, often in a metabolic frame (Fomon, 1974), for up to 72 hours may have on nitrogen retention in an infant. It has been suggested that nitrogen retention may be reduced under such conditions (Scrimshaw, 1977), which may lead to an underestimation of the digestibility of dietary nitrogen. In support of the methods and results of the work described in chapter 2, an apparent faecal digestibility of 95% was determined for the nitrogen in human milk after nitrogen retention had been measured in breast-fed infants in a home environment using diaper collection of faeces over a 7 day period (Prentice *et al.*, 1987). The findings of Prentice *et al.* (1987), together with those reported in chapter 2 of this study, appear to highlight the need for re-evaluation of the methods presently used to determine nitrogen retention in infants.

Small but significant differences were found, in the study reported in chapter 2, between the infant and piglet for the apparent faecal digestibility of total nitrogen, dry matter and organic matter. Faecal digestibility coefficients for most of the amino acids were not significantly different between the two species, however, suggesting that digestion of protein is similar in the three-month-old human infant and three-week-old piglet, at least for a highly-digestible milk protein. Comparison of protein digestion in the human infant and piglet using a wider range and quality of dietary proteins may be useful in future investigations of the piglet as a model animal for the human infant.

The inherent limitations of faecal digestibility values also need to be considered when a comparison of the infant and piglet is made. The piglet appeared to digest the non-amino acid fractions of the infant formula to a greater extent than the infant, possibly due to a more active microbial population in the piglet's large intestine. Comparison of protein digestion in the human infant and piglet at the ileal level (via infants and piglets with ileostomies) may be more appropriate. Further validation of the piglet model, therefore, could include such a comparison, although the 'non-physiological' nature of an ileostomy and the condition that necessitated the ileostomy in the infant would need to be taken into consideration.

Assuming that the piglet is a suitable model for the human infant, the piglet can be used to determine the ileal digestibility of amino acids in human milk. The ileal assay, however, relies on the assumption that amino acids are not absorbed in nutritionally significant amounts from the large intestine. While this is generally

accepted as being true in older pigs (Zebrowska, 1973), there is some *in vitro* evidence to suggest that the piglet's large intestinal epithelium is capable of absorbing amino acids (James and Smith, 1976; Jarvis *et al.*, 1977). No indication was given in these latter studies, however, as to whether the amounts of amino acid absorbed were of nutritional benefit. An *in vivo* investigation of the absorption of amino acids from the large intestine of the piglet (chapter 3) revealed, however, that lysine and methionine are not absorbed in nutritionally significant amounts from the proximal colon of three-week-old piglets. Given the similarities in the hindgut epithelium of the infant and piglet at the comparable developmental stage of peak lactation in the dam (Moughan *et al.*, 1992), it is assumed that this also applies to three-month-old human infants . Application of the same technique as that used in chapter 3, to investigate the absorption of amino acids from the large intestine of younger piglets (ie. 1-7 days of age), would be interesting, and may be important in the establishment of the younger piglet as a model for the premature infant as suggested by Moughan *et al.* (1994).

Accepting that the piglet is an appropriate model for the human infant for studying protein digestion and that the ileal digestibility assay can be used with confidence in young milk-formula-fed piglets, it was possible to proceed with the ultimate aim of the overall study, namely determining the digestible amino acid composition of human milk. Fundamental to this aim, however, was accurate determination of the gross amino acid composition of human milk, for it is this profile that is corrected for digestibility. To this end, a series of two experiments were conducted. Firstly, a method was established for correcting for amino acid loss in a purified protein. This involved the application of a non-linear compartmental model (Model I) that describes the simultaneous processes of amino acid yield and decay that occur during acid hydrolysis of a protein prior to amino acid detection (chapter 4). In addition to serine and threonine, which are known to be acid-labile, it became apparent that most amino acids in a protein experience some degree of loss during hydrolysis. Of particular note was the loss rate exhibited by cysteic acid, which was larger than that shown for serine. Consequently, the standard single hydrolysis interval (usually 24 hours) used to hydrolyse proteins will, in some cases, introduce a significant error in determination of the amino acid composition of a protein. Non-linear regression using data derived from multiple hydrolysis intervals will allow accurate estimation of the amino acid composition of a protein, with 10 hydrolysis intervals providing an acceptable compromise between accuracy and the cost of analysis.

Biological substances, such as human milk, often contain a mixture of different proteins, and amino acids in free form. Therefore, in a second experiment (chapter 5), a new model was derived (model II) that included a component to describe the loss of amino acids present in free form prior to hydrolysis. Models I and II were compared, and it was concluded that although using Model I will not lead to the introduction of any large degree of bias in determination of the amino acid composition of a sample contained both protein and free amino acids, Model II gave more accurate estimates of the amino acid content.

Correcting for amino acid losses during acid hydrolysis is deserving of increased attention, as Models I and II have only been applied to lysozyme and human milk, respectively. Further research is required into the loss rates of amino acids in different types of proteins, in the presence of different contaminants, and under different hydrolytic conditions such as those suggested by Gehrke *et al.* (1985) and Zumwalt *et al.* (1987). In addition, application of the non-linear model to correct for loss of tryptophan during alkaline hydrolysis would be interesting, particularly as this amino acid is of considerable significance in human nutrition.

When the model parameters for the hydrolysis and loss rates for each amino acid in a specific protein have been determined, they can be used to correct subsequent hydrolysates of that protein, measured at any given time, provided the conditions of hydrolysis are identical to those used when determining the parameters. It can be argued that such corrections are not necessary if the amino acid data are to be used in a relative manner, for example in determining the digestibility of a protein. This would only be valid, however, if the rate of amino acid loss was identical for different types of protein. Comparison of the loss rates for amino acids in either a purified protein (chapter 4) or a biological sample containing several different proteins (chapter 5) revealed, however, that amino acid loss rate is dependent on the type of protein. Rowan *et al.* (1992) also found that for several amino acids, the loss rate of each amino acid differed between diet, ileal digesta or faecal samples. Thus, when absolute accuracy is required, correction should be made for amino acid losses during hydrolysis.

The methodology established in the studies described in chapters 2, 3, 4 and 5, was used to determine the digestible amino acid composition of human milk (chapter 6). The study was conducted in two parts. Part I involved determination of the amino acid composition of human milk with correction for amino acid losses during

hydrolysis, using estimates of the non-linear model parameters derived in chapter 5. The second part involved determination of true ileal amino acid digestibility coefficients for human milk using the piglet as a model animal for the human infant. The amino acid composition of human milk determined in the present work was similar to previously reported estimates, excepting cysteine which was found to have a considerably higher concentration than previously reported. Had no correction been made for losses of cysteic acid during acid hydrolysis, the present cysteine value would have been similar to literature estimates.

The overall digestion of amino acid nitrogen reported in chapter 6 was higher than would be expected based on levels of the immune proteins found in the faeces of breast-fed infants (see Table 1.6, page 41). Intact immune proteins found in the faeces of breast-fed infants may be of endogenous rather than milk origin (Haneberg and Tonder, 1973; Koutras and Vigorita, 1989), however, and their presence in the faeces of breast-fed infants would lead to an underestimation of the digestibility of human milk proteins. Whether excretion of endogenous immune proteins into the gastrointestinal tract of breast-fed infants is stimulated by a factor in human milk is unknown, although research with premature infants appears to support such a theory (Goldman *et al.*, 1990). Correcting the ileal amino acid flows determining in the piglets for endogenous amino acid excretion would have accounted for this possibility had it occurred in the piglet. Thus, the the digestibility coefficients determined using the piglet may be a more accurate estimate of the digestible protein content of human milk.

Similarities in the digestive anatomy and physiology of the human infant and piglet (Moughan *et al.*, 1992), and in the digestion of dietary protein (refer chapter 2), provide support for the piglet as a model animal for studying aspects of protein digestion in the human infant. It is possible, however, that the piglet may have been more efficient than the breast-fed infant at digesting protein to the end of the small intestine, or there may be have been more proteolytic bacteria present in the upper digestive tract of piglets compared to human infants, which could in part, explain the higher than expected digestibility of amino acid nitrogen reported in chapter 6. These possibilities can only be investigated, however, by comparison of the piglet and human infant at the ileal level.

Determining true ileal amino acid digestibility coefficients for human milk in the study reported in chapter 6, involved the collection of ileal digesta samples using the slaughter method. One of the main criticisms of this method concerns the potential

difficulty in obtaining representative samples of digesta. A desirable objective for assays using the slaughter technique is to maintain a uniform flow of digesta through the gastrointestinal tract. In the present study, the piglets were fed hourly for up to 48 hours prior to ileal digesta collection, and it was assumed that an even flow was maintained. The size of sample collected in the present study was of some concern, however, with the ileal digesta samples collected from the human-milk-fed piglets being smaller than expected based on preliminary work with formula-fed piglets (AJ Darragh, unpublished). Future work, using the piglet as a model animal, may need to include cannulation of the piglet's terminal ileum, or even construction of an ileostomy in the piglet. This would increase the sample size, which in turn would allow more detailed chemical analysis of the digesta collected.

The indigestible marker ($Cr_2O_3$) used in the study described in chapter 6, is commonly adopted as a marker for dry matter only. There is the possibility that the marker did not flow homogeneously through the piglet's gut with the digesta, introducing error into the estimation of digestibility. However, preliminary studies (AJ Darraghh, unpublished) with three-week-old milk-fed piglets suggested that this was not the case, at least to the beginning of the duodenum. Markers that associate with both the solid and liquid phases of a diet such as human milk, may need to be considered in future studies, although these sorts of markers are not without their own limitations (Kotb and Luckey, 1972; Weström *et al.*, 1984).

Although an overall mean true ileal amino acid nitrogen digestibility of 95% suggests that in practical terms the protein in human milk is highly digestible, there is still an indication that specific proteins in human milk may be at least partially resistant to digestion. This is apparent when true ileal digestibility values for the amino acids in human milk are compared with true ileal digestibility values for the amino acids in a bovine-milk-based formula (refer Table 8.2) determined in three-week-old piglets (chapter 7). The greatest differences between the amino acid digestibility of human milk and the infant formula occurred for those amino acids that are present in higher proportions in the immune proteins (Harzer and Bindels, 1987). The lower digestibility of these amino acids suggests that the immune proteins are at least partially resistant to digestion. Lysine in the infant formula appeared to be less digestible than the lysine in human milk, suggesting that a small degree of heat damage of the protein in the infant formula occurred during processing.

**Table 8.2.**

*The true ileal digestibility[•] of amino acids in human milk[+] and a bovine-milk-based infant formula[#]*

| Amino Acid | Human Milk | Infant Formula |
|---|---|---|
| Amino Acid Nitrogen | 95 | 101 |
| Aspartic Acid | 95 | 101 |
| Threonine | 86 | 99 |
| Serine | 95 | 102 |
| Glutamic Acid | 98 | 99 |
| Proline | 92 | 100 |
| Alanine | 95 | 101 |
| Valine | 90 | 100 |
| Isoleucine | 98 | 100 |
| Leucine | 99 | 101 |
| Tyrosine | 100 | 102 |
| Phenylalanine | 93 | 102 |
| Histidine | 95 | 101 |
| Lysine | 98 | 95 |
| Arginine | 101 | 103 |
| Methionine | 100 | 101 |

• The true ileal digestibility values were calculated from the dietary ratio of nutrient to chromium, relative to the corresponding ratio in the ileal digesta, and were corrected for endogenous amino acid excretions.
+ From Table 6.5, page 173.
# From Table 7.2, page 191.

Correcting the gross amino acid composition of human milk for digestibility using the digestibility value for amino acid nitrogen only, would result in a reduction in the total amount of each amino acid estimated to be required by the infant, but would not have altered the pattern of amino acids estimated to be required by the infant. It is obvious, however, that this is unsatisfactory, as each amino acid has a different digestibility. Therefore, it is important to use the individual amino acid digestibilities to correct the gross amino acid composition of human milk. In fact, when this is done, the pattern of amino acids thought to reflect the infant's requirements changed considerably (refer Table 6.6, page 178). This has significant implications, not only with regard to estimation of the infant's requirements, but also in the evaluation

**Table 8.3.**

*The profile[*] of digestible essential[+] amino acids in human milk, the currently recommended profile of amino acids required by the human infant, and the amino acid profile in bovine milk protein, and in soya protein*

| Amino Acid | Digestible Human milk Pattern[#] | Recommended Pattern[§] | Bovine Milk Protein[¶] | Bovine Casein[¶] | Bovine Whey Protein[¶] | Isolated Soyabean protein[✿] |
|---|---|---|---|---|---|---|
| Lysine | 100 | 100 | 100 | 100 | 100 | 100 |
| Histidine | 37 | 39 | 34 | 35 | 22 | 42 |
| Isoleucine | 79 | 70 | 76 | 68 | 69 | 87 |
| Leucine | 148 | 141 | 125 | 125 | 112 | 144 |
| Methionine/Cysteine | 65 | 64 | 43 | 37 | 49 | 59 |
| Phenylalanine/Tyrosine | 122 | 109 | 125 | 133 | 74 | 164 |
| Threonine | 65 | 65 | 61 | 55 | 81 | 64 |
| Valine | 75 | 83 | 82 | 81 | 69 | 94 |

* Expressed relative to lysine = 100 units.
\+ Excluding tryptophan.
\# Corrected based on the true ileal amino acid digestibility of human milk.
§ FAO/WHO/UNU (1985).
¶ From Heine *et al.* (1991).
✿ From Moughan *et al.* (1990).

of protein quality in foods destined for consumption by children 1 year of age or younger, as it is the amino acid composition of human milk that is used as a standard for protein quality evaluation (FAO/WHO, 1990). This is clearly demonstrated in Table 8.3, where the digestible amino acid composition of human milk, the pattern of amino acids (based on the gross amino acid composition of human milk) currently thought to be required by human infants (FAO/WHO/UNU, 1985), and the amino acid composition of different proteins used in infant formulation are given. Scoring of the proteins relative to the digestible or gross amino acid patterns of human milk (refer chapter 7 for method) would have resulted in different ratings for the individual proteins, particularly for leucine, valine, and the aromatic amino acids.

Whether an infant formula will be limiting in any of the essential amino acids required by the infant is generally not an issue, for as discussed in chapter 7, formulas often contain more protein than human milk to take into account a supposedly lower protein quality and digestibility. As reported in chapter 7 and summarised in Table 8.2, the amino acids in formulas are almost completely digested, however, and providing more protein in a formula to account for a lower digestibility is unnecessary. In addition, there does not appear to be any reason for maintaining the high levels of protein in infant formulas because of inferior protein quality. In fact, amino acid excess would appear to be of more concern. Therefore, it can be argued that the protein content of infant formulas can be decreased to a level similar to that of the digestible protein content of human milk. There is considerable debate, however, as to whether the protein content of infant formulas should be reduced. Some believe that reducing the protein content of infant formulas would remove the margin of safety necessary to buffer for lower protein quality. Others believe that the higher growth rates observed in formula-fed infants compared to breast-fed infants (Heinig *et al.*, 1993) is desirable and should be maintained.

This leads into a whole new dimension of debate, however, as to what are the minimal, optimal and maximal growth rates for infants. In domestic animals the criteria for optimal performance are easily defined, and as such the quantity of nutrients required by the animal to achieve such criteria can be readily determined. In growing animals the pattern of amino acids required for protein deposition is largely set by the amino acid composition of the proteins being deposited (Reeds and Hutchens, 1994), and the total amounts of amino acids consumed will dictate the growth rate achieved by the animal.

Those in the field of infant nutrition, who accept that breast milk is the standard by which all infant feeding should be based, believe that the growth rate achieved by the breast-fed infant is optimal. Others, however, suggest that on an evolutionary basis lactational output is a compromise between the infant's requirements and the depletion of maternal protein stores (Blaxter, 1961; Fomon, 1986), and that the breast-fed infant is not growing optimally. Indeed, it has been speculated that the amino acid intake of the breast-fed infant represents the infant's minimal requirement for amino acids (Fomon, 1991). It is unlikely that the average intake of amino acids in a population of breast-fed infants represents the minimum requirement, however, as this would imply that 50% of the population were receiving less than the recommended requirement. It is possible, however, that the growth rate achieved by breast-fed infants is not optimal.

Assuming that the breast-fed infant's growth rate is not optimal, it would appear necessary to determine the maximal growth rate and to determine whether this is optimal. Possibly the only way to assess whether the faster growth rate of formula-fed infants is beneficial or harmful is to look at the effects of increased protein intake consequent upon formula-feeding. Casey (1989) suggested that these could be assessed at three levels, namely; the short-term, intermediate-term and long-term consequences.

Firstly, there are the short-term consequences, which relate to the immediate response in the infant to increased protein intake. Alteration of the plasma amino acid concentrations of formula-fed infants compared to breast-fed infants is the most obvious short-term outcome. While this area has been the subject of intensive research over the last decade, and significant differences in the plasma amino acid concentrations of formula-fed and breast-fed infants have been documented (Lindblad *et al.*, 1978; Järvenpää *et al.*, 1982; Räihä *et al.*, 1986; Lönnerdal and Chen, 1990), whether these altered plasma amino acid concentrations have any detrimental effect on the healthy full-term infant is yet to be elucidated (Young and Pelletier, 1989). Intermediate-term consequences include the effect of protein intake on growth rate, and general health of the infant during the period that they are milk-fed. In a comprehensive study of infants over a period of 12 months, Heinig *et al.* (1993) found that while formula-fed infants grew faster than breast-fed infants, the breast-fed infants gained more weight and lean body mass per gram of protein intake compared to formula-fed infants. This suggests that there is a considerable metabolic waste of dietary protein in the latter group which may place excessive strain on the infant's

catabolic metabolism, particularly the renal system (Berg *et al.*, 1987). The study of Heinig *et al.* (1993) also found no evidence of any functional advantage to the more rapid growth rate of the formula-fed infants. This is supported by an earlier longitudinal study of children from birth up to seven years of age which found no differences in weight or fatness of children according to the type of infant feeding (Birkbeck *et al.*, 1985). Studies on the long-term effects of type of infant feeding on fatness and serum cholesterol levels, indices of risk of cardiovascular disease and other degenerative diseases later in life have been reviewed (Dobbing, 1985; Hamosh and Hamosh, 1987). Due to the numerous factors that can exert an influence on these conditions, however, comparison of formula-feeding versus breast-feeding in this sense has generally been inconclusive.

It would appear, therefore, that while there is no real indication that the higher growth rate achieved by formula-fed infants is harmful, there is no evidence to suggest that it is beneficial. Until further research in this area is conducted, the composition of human milk and performance of the breast-fed infant will remain the most obvious standards by which aspects of infant nutrition can be assessed.

Evidence found in the literature and in the present study suggests that the digestible rather than gross amino acid composition of human milk would be a better basis for determining amino acid requirements for the human infant. Although it is possible to estimate the digestible amino acid composition of human milk using apparent amino acid digestibility values for human milk determined over the entire digestive tract of infants, determining the digestibility of amino acids in human milk to the end of the small intestine, with correction for the endogenous excretion of amino acids, is considered more appropriate. The work described in the present study provides a preliminary estimate of the digestible amino acid composition of human milk, and this estimate contributes towards determining the amino acid requirements of the human infant.

## 8.2 References

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