NEONATAL SULFUR AMINO ACID METABOLISM

M.A. Riedijk

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Zwavel aminozuur metabolisme in de neonatale periode

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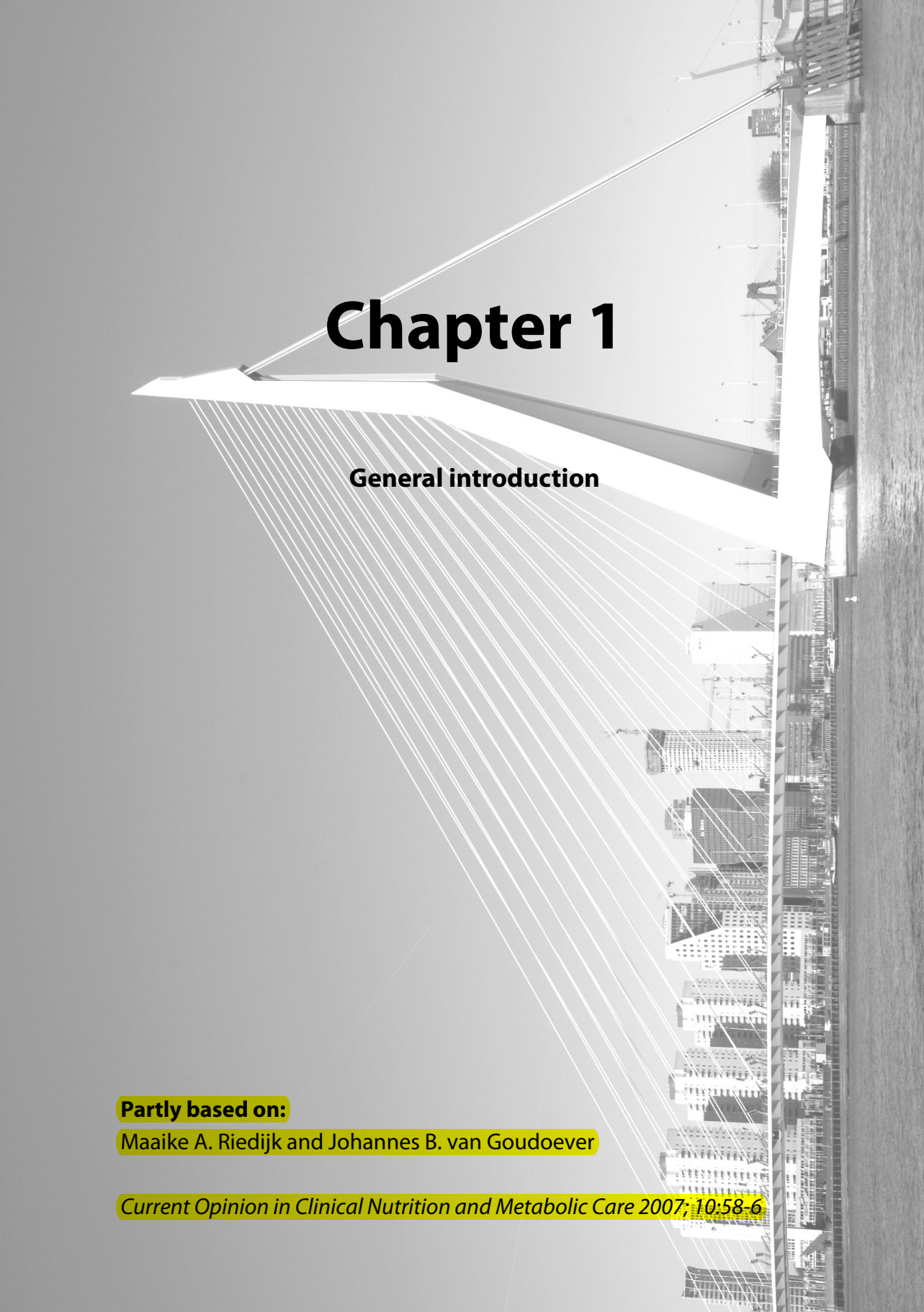

Chapter 1

General introduction

Partly based on:

Maaike A. Riedijk and Johannes B. van Goudoever

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GENERAL INTRODUCTION

At birth, infants can be classified either by gestational age (GA) or by weight. Neonates born < 37 weeks are classified as preterm, < 28 weeks as very preterm and < 26 weeks as extremely preterm infants. Prematurely born infants can also be classified by birth weight as follows (1):
- Low birth weight (LBW) < 2500 g
- Very low birth weight (VLBW) < 1500 g
- Extremely low birth weight (ELBW) < 1000 g

However, both classifications are used arbitrary in the clinical setting and obviously the infant born very prematurely has most likely a birth weight < 1500 g.

Over the past 25 years life support in the neonate born prematurely has improved tremendously and has created major advantages in medical treatment possibilities and survival. Nowadays it is generally accepted to treat the very preterm infant and even the extremely preterm infant since survival rates for these infants are still increasing. During the eighties mortality rate of preterm infants born at GA of 25-27 wk was 52.1% (n = 169) and these rates decreased dramatically to 31.8% (n = 88) in 1990 (2). The mortality in infants at 28-31 wk GA decreased as well, although less impressive from 15.2% (n = 559) to 11.3% (n = 388) (2). On the flip side of the coin, morbidity in these infants is still high with 64% in infants born at GA 22-25 wk at the time of discharge (3). Another recent study showed that the survival chance of infants born with GA < 26 wk free from serious neonatal morbidity at the time of hospital discharge was < 15% (4). Overal surival rate for infants born with GA ≤ 26 wk is 39-54% (3, 4) and 89% for infants born with GA < 32 wk (2). The National Institute of Child Health and Human Development Neonatal Research Network showed in 4438 infants weighing 501 to 1500 g at birth that 84% survived until discharge (1). Survival to discharge increased with birth weight and showed improvement, particularly in infants weighing < 1000 g. The incidence of chronic long disease (23%), necrotizing enterocolitis (7%) and severe intracranial hemorrhage (11%) did not change. However, 99% of the infants weighing < 1000 g showed poor postnatal growth. Very recently Saigal et al. (5) reported in a study on the long-term outcomes on ELBW young adults that most former ELBW infants have overcome their earlier difficulties to become functional young adults. Another study (POPS cohort at 19 years of life) supported this concept of coping behavior of ex preterm infants in later life. They showed that the vast majority (> 95%) of young adolescents born premature were very satisfied with their lives and a just a minority (< 10%) suffered from major disabilities (6).

Along with the major advances in life support measures, nutrition has attracted more attention in the care of ELBW and VLBW infants. However, to date neonatologists are more focused on mechanical ventilation and infections. Nutrition is still considered as relatively unimportant. Until recently, it was believed that premature infants do not tolerate much parenteral feeding and total parenteral nutrition was standard regimen after a couple of days post partum.

Birth involves the transition from intrauterine to extrauterine life and requires rapid

adaptation of several organs. Before birth, the fetus receives a continuous supply of nutrients from the placenta and at birth this is suddenly terminated and the gastrointestinal tract needs to adapt to enteral feeding. The final maturation of the gastrointestinal tract for enteral nutrition is shortly before or at term. Preterm birth is associated with immaturity of the gastrointestinal tract involving complications such as immature digestion, absorption and motility. It seems that enteral feeding in infants born prematurely is mandatory for the development of the gastrointestinal tract (7, 8). The clinical condition of the preterm infant frequently allows mainly parenteral feeding and hardly enteral feeding during the first week. Parenteral feeding is associated with several complications, including increased risk of infection, mucosal atrophy and cholestatic jaundice. Introduction of enteral feeding is therefore required as soon as possible. Also, enteral feeding stimulates postnatal gastrointestinal development. However, enteral feeding in these infants is associated with detrimental morbidity, like necrotizing enterocolitis (9).

Early nutrition is pivotal for preterm infants' survival but has also profound influence on their later developmental and intelligence outcome (10). It is of utmost importance to offer optimal nutrition to enhance growth. However, rapid early growth in term infants born small for gestational age is related to the development of metabolic syndrome (hypertension, dyslipidemia, obesity and insulin resistance) and non-insulin dependent diabetes later in life (11, 12). Whether this phenomenon concerns the preterm infant is under debate. Controversial results have been published whether preterm birth is related to insulin resistance (13, 14). Hofman et al. (14) showed in children 4 to 10 years old who were born prematurely, whether their birth weight was low or appropriate, that insulin resistance was presented. As compared with control children who were born at term and appropriate for gestational age, infants who had been born prematurely had a reduction in insulin sensitivity, irrespective of their birth weight. These findings should be interpreted with care because epidemiologic studies have not yet indicated that there is an increased incidence of type 2 diabetes mellitus or other manifestations of the fetal-programming hypothesis among long-term survivors of prematurity. Besides, the era of increasing survival of preterm infants is too short to examine long-term consequences of prematurity. The development of diabetes type 2 could also be induced by entirely different mechanisms, rather than those related to intrauterine growth restriction. In addition, the authors comment little on the effect of interactions between birth weight and early postnatal growth on subsequent insulin sensitivity. Subsequently, it was speculated that insulin resistance is associated with early nutrition and diet composition (15). It is therefore important to determine the specific nutrient requirements, i.e. carbohydrates, protein and fat.

The goal in estimating the nutritional needs of the preterm infant is not only to provide the quantity and quality of nutrients needed to achieve fetal rates of tissue growth and nitrogen accretion (16), but also to provide a functional development as the infant would have in case he was not born prematurely. In view of the protein quantity, in the past years it has become clear that the minimum and maximum protein concentration in preterm infant formula

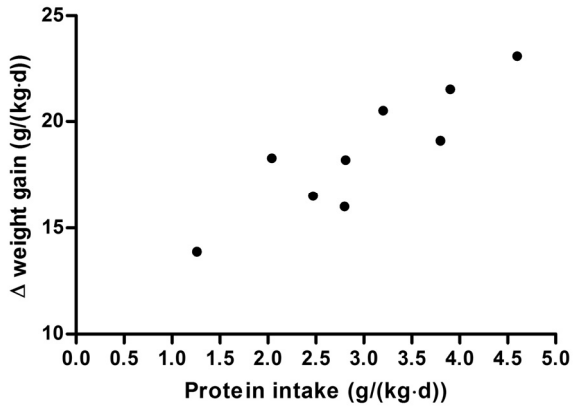

Figure 1. Weight gain is dependent on protein intake (18-21).

should be 2.5 g/100 kcal and 3.6 g/100 kcal, respectively (17). A new guideline will be published, advising to administer an average protein intake of 3.8-4.5 g/(kg·d) to infants weighing less than 1800 g (2008 ESPGHAN guideline on enteral preterm nutrition). Inadequate amino acid intake impairs protein synthesis, which is pivotal for growth. Protein intake is related to weight gain as depicted in Figure 1.

An optimal intake of amino acids is thus necessary for adequate protein accretion in neonates but might also prevent diseases. However, the specific amino acid requirements for ELBW and VLBW infants to meet their metabolic needs are poorly defined.

AMINO ACIDS

Amino acids are molecules where the amino and carboxyl groups are attached to the same carbon atom, which is called the α–carbon. The various alpha amino acids differ in which side chain is attached to their alpha carbon. α-Amino acids are the building blocks for proteins. There are twenty standard α–amino acids used by cells in protein biosynthesis and these are specified by the general genetic code The genetic code is the set of rules by which information encoded in genetic material (DNA or RNA sequences) is translated into proteins (amino acid sequences) by living cells. These twenty amino acids can be either biosynthesized from simpler molecules or must be obtained from the diet. The carbon skeleton of indispensable amino acids cannot be synthesized endogenously in the body and must therefore be supplied by the diet. Dispensable amino acids can be synthesized de novo in the human body from other amino acids. The eight indispensable amino acids in the human are: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The dispensable amino acids are: alanine, arginine, asparagine, aspartate, cysteine, histidine, glutamate, glutamine, glycine, proline, serine and tyrosine. However, some dispensable amino acids are considered conditionally indispensable during specific circumstances, e.g. rapid growth or critical illness. Endogenous synthesis then will

temporarily not be sufficient to meet the requirement. Conditionally dispensable amino acids during early development, because of the biochemical immaturity of the preterm infant include arginine, cysteine, glutamine, glycine, histidine and tyrosine (22-24).

ESTIMATING REQUIREMENTS

How to estimate the specific nutritional requirements in preterm infants? Different methods have been used to estimate dietary requirements, eg the nitrogen balance method, weight gain rate, plasma amino acid patterns and the factorial approach. Snyderman et al. (25) were in 1953 the first to describe requirements for specific amino acids in infants. They reported amino acid requirements in infants (Table 1) by means of nitrogen balance and weight gain (26-31).

Table 1. The minimal requirements of certain amino acids for infants (22-27).

Amino acid	Estimated minimal requirement mg/(kg·d)
Histidine	17-35
Isoleucine	79-126
Lysine	90-105
Methionine	32-49
Phenylalanine	63-91
Threonine	45-87
Valine	85-105

Nitrogen balance is the difference between the amount of nitrogen consumed and the total nitrogen losses (including urine, feces and miscellaneous such as skin, hear, sweat, breath). The principle of the nitrogen balance method is to increase the test amino acid intake from deficient to adequate, whereupon nitrogen balance and growth rate will progressively increase until the requirement is reached. A disadvantage of this method is that it is difficult to determine the nitrogen losses accurately. In most studies these losses are measured only in urine and feces but not through skin, hear and sweat and often some urine or feces are lost and therefore not included in the balance. On the other hand, nitrogen intake is often not accurate estimated but overrated because of spilling or vomiting. A combination of these errors results in overestimating or underestimation (assuming that the provided intake is truly consumed) of the nitrogen retention. Another drawback is that it requires 7 to 10 days for the body to adapt to the nitrogen quantity of test diet (32). Currently, this method is not applicable anymore to estimate the requirements for preterm infants because present-day practice will not accept to maintain neonates on either deficient or excess amino acid intakes for a minimum of 7 days.

A different method to determine requirements is to measure biological or physiological responses to graded intakes of the nutrient under study. Several studies have been done to investigate the profile of plasma amino acid concentrations in preterm infants during different feeding regimens, ie parenteral, human milk or formula feeding. The goal was to compare amino acid pattern in these infants to term breast-fed infants (33-35). However, it is not plausible to compare the infant born prematurely to those born term because of the different metabolic needs and developmental stage.

At present, it is generally believed that the growth rate of the preterm infant should mimic at least the growth rate of the intrauterine fetus at the same gestational age (36), but that at the requirement level the infant should be provided with enough nutrients to sustain a functional development similar to that of a normal newborn (ESPGHAN 2008). Nutrient requirements are currently based on the factorial method (37, 38). In this method the obligatory nutrient losses (maintenance requirement) is added to the nutrients required for protein synthesis (growth requirement) (39). The factorial model to define the protein requirements for preterm infants is based on fetal body composition during normal intrauterine development. Body composition data derived by chemical analysis from body carcass of stillborn preterm infants are quite old (40). Ziegler (41) has used various of these data to create a reference fetus. However, these analysis are not completely reliable because they were done when the gestational age could not be exactly determined by advanced techniques and was completely dependent on maternal history. Another difficulty is that the reason for preterm birth was not recorded and differentiation in intrauterine growth restriction was not made. Subsequently, these date were re-analyzed by Sparks (42) and corrected for these confounders. Nonetheless, the fact is that these data are still old and for some amino acids data are not available.

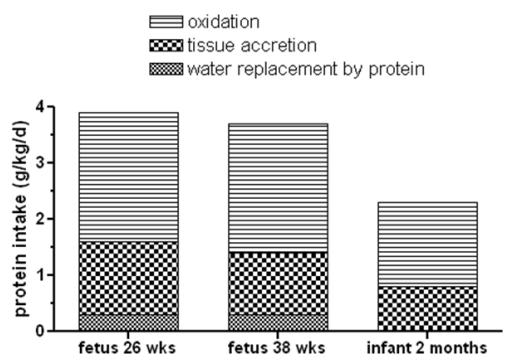

Figure 2. Fetal data from carcass analysis. Data reviewed by te Braake et al. (43)

Recently, a new method to determine dispensable amino acid requirements have been developed: the indicator amino acid oxidation (IAAO) (44). Kim et al. (45-47) were the first to describe the determination of amino acid requirements of young piglets using the IAAO. This method is based on measurements of amino acid oxidation. The indicator is a stable isotopically labeled indispensable amino acid and is different from the amino acid under

study. The indicator needs to follow the same pathway as the identical unlabeled dispensable amino acid. At the same time, oxidation needs to result in the release of labeled CO_2 and without intermediary metabolism, such as methionine. The indicator is independent of the different intake levels of the test amino acid. The IAAO method is based on the assumption that the partition of any indispensable amino acid between oxidation and protein synthesis is sensitive to the level of the most limiting amino acid in the diet (44). If an indispensable amino acid is deficient in the diet, this will limit overall protein synthesis and all other amino acids will be subsequently oxidized. Increasing the dietary intake of the test amino acid will linearly decrease oxidation of the indicator until requirement of the test amino acid is met. A limitation of this method is the necessity of providing all amino acid in excess, except for the one under study. Consequently, insufficient intake of indispensable amino acids could result in another indispensable amino acid than the test amino acid being limiting for protein synthesis. Leucine, lysine and phenylalanine are mainly used to serve as an indicator. The choice of the best indicator is still under debate; in piglets phenylalanine was demonstrated to be the best indicator (48), and a study in humans demonstrated that leucine was not a good choice as the indicator (49). In our view, phenylalanine is still the best choice to use as the indicator. After Kim et al. (45-47) introduced the IAAO, Zello et al. (50) introduced the method to use in human studies. With the use of this method the requirements of tryptophan, threonine, lysine and sulfur amino acid of human adults have been determined (51-54). Further adaptations have led to a non-invasive method for measuring indispensable amino acid requirements in compromised populations (55-57). Despite the minimally invasive method to determine requirement, only the tyrosine requirements in parenterally fed preterm infants have been established so far (58). It is of great importance to determine the specific indispensable amino acid requirements in preterm infants to meet their needs, because temporary metabolic and physiological immaturity of these infants often leads to a delayed onset of adequate endogenous synthesis of amino acids. Although there is a vast amount of guidelines recommending total protein intake for preterm infants, hardly any data are available on specific amino acid requirements.

SULFUR AMINO ACIDS

Sulfur amino acids have gained much interest in the last couple of years mainly because of the link to cardiovascular disease (59) and neurological diseases, e.g. Alzheimer (60). More recently, it has been shown that homocysteine also plays an important role in pregnancy complications (61, 62) and neural dysfunction (63, 64). The sulfur amino acids in mammals are: methionine, homocysteine and cysteine. However, methionine and cysteine are the only proteinogenic sulfur amino acids. Homocysteine is not incorporated in proteins and functions as an intermediary in methionine metabolism. Sulfur is an essential element of all living cells. The free sulfur moiety in cysteine is important in the formation of disulfide bonds, for instance in stabilizing protein structures. One cysteine molecule can form a disulfide with another cysteine molecule and is called cystine.

Methionine is the only indispensable sulfur-containing amino acid in humans because the carbon skeletal cannot be synthesized in the human body. It is an important methyl donor for several biosynthetic processes and its sulfur is essential for cysteine synthesis. Cysteine on the other hand, is a dispensable sulfur amino acids in (adult) humans because it can be synthesized de novo from methionine via homocysteine and serine. As mentioned previously, it is believed that cysteine is a conditionally indispensable amino acid in infants born prematurely. Enzymatic pathways involved in endogenous cysteine synthesis are absent or activity is limited (65, 66). Cysteine is a key amino acid for premature infants since it is the precursor for glutathione, which plays a major role in the antioxidant defense system. Some complications of premature birth are based upon oxidant damage, like retinopathy of the premature (ROP) and bronchopulmonary dysplasia (BPD).

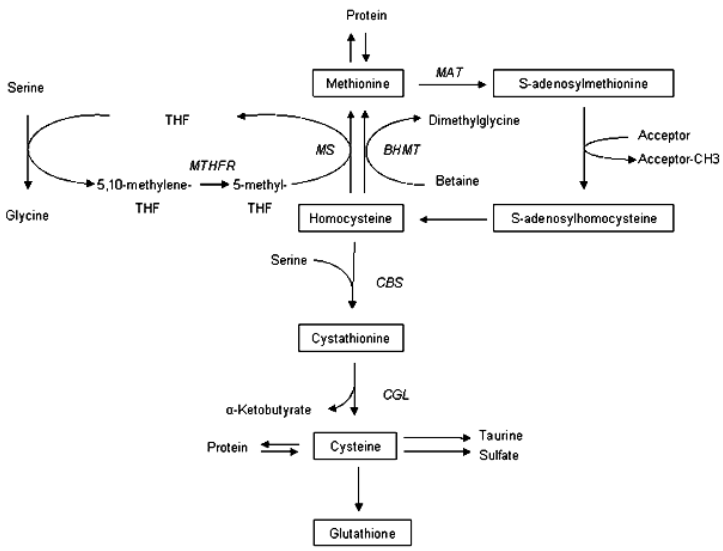

Figure 3. Major pathways of sulfur-containing amino acids. MAT methionine adenosyltransferase, MS methionine synthase, BHMT betaine-homocysteine methyltransferase, CBS cystathionine ß-synthase, CGL cystathionine γ-lyase, MTHFR methylenetetrahydrofolate reductase, THF tetrahydrofolate.

Sulfur amino acids pathways

The major metabolic pathways of sulfur amino acids are depicted in Figure 3. Methionine is converted to homocysteine via *S*-adenosylmethionine (SAM) by transmethylation. SAM is the major methyl donor in cells; methylations are essential for biosynthesis of various cellular compounds including, carnitine, creatine, phospholipids, epinephrine, proteins, DNA and RNA (67). Homocysteine is not used for protein synthesis but is a key compound in the regulation of methionine and cysteine plasma concentrations. It is removed by remethylation or transsulfuration. Remethylation is the reformation of methionine, which is catalyzed by the enzyme methionine synthase and conserves a methyl group from either ^{5}*N*-methyl-tetrahydrofolate or betaine. Methionine synthase (EC 2.1.1.13) utilizes methylcobalamin as a prosthetic group, and requires vitamin B12. The methyl group utilized by methyl synthase is

provided from the folic acid pool. Methylenetetrahydrofolate reductase (EC 1.5.1.20), which reduces 5,10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate, contains flavin adenine dinucleotide (FAD) as a prosthetic group. FAD is a coenzyme derived from riboflavin (vitamin B2) (68). Homocysteine can also be irreversibly catabolized in the transsulfuration pathway yielding cysteine. The irreversible transsulfuration pathway involves the enzymes cystathionine β-synthase (E.C. 4.2.1.22) and cystathionine γ-lyase (E.C. 4.4.1.1), and regulates cysteine synthesis. If methionine is in excess, homocysteine is condensed with serine to form cystathionine, which is catalyzed by the enzyme cystathionine β-synthase (CBS). Cystathionine is then hydrolyzed by cystathionine γ-lyase to form cysteine. This includes transfer of the sulfur moiety from homocysteine to the carbon skeleton of serine. CBS is the rate-limiting enzyme for cysteine synthesis and decreased activity might lead to insufficient cysteine production to meet the requirements. The carbons derived from homocysteine are catabolized to α-ketobutyrate, which enters the tricarboxylacid (TCA) cycle. The enzymes involved in the transsulfuration pathway require pyridoxal 5'phosphate (PLP), which is the active co-enzyme form of vitamin B6. It is not surprising that the concentrations of vitamin B2, B6, B12 and folic acid have a major impact on sulfur amino acid metabolism resulting in elevated homocysteine concentrations.

Cysteine

There are different pathways of cysteine utilization. First, it plays a key role in cellular protein function and redox status because of its thiol (-SH) moiety (69). Furthermore, it is a one of the three amino acid components of the tripeptide glutathione (glutamate-cysteine-glycine), a major intracellular antioxidant. Also, it is a precursor for the production of taurine as well as coenzyme A and sulfate. These metabolites have important physiological roles in detoxification, bile acid formation, membrane stabilization and neurotransmission (70). In humans almost all cells are capable of transmethylation and remethylation, thought transsulfuration is restricted to certain tissues. Several in vitro studies showed that CBS and cystathionine γ-lyase activity was predominantly reserved to the liver (71-73).

As mentioned previously, cysteine is believed to be a conditionally indispensable amino acid in preterm infants caused by biochemical immaturity of CBS. Sturman et al. (65) and Gaull et al. (66) were the first to report in vitro results about CBS activity in preterm infants. They found that CBS activity was absent in fetal liver tissues, suggesting that cysteine might be a conditionally indispensable amino acid in preterm infants due to absent CBS activity. Zlotkin et al. (72) also reported limited enzyme activity in liver tissues of deceased infants born prematurely and found that this increased rapidly after birth, reaching mature levels at the age of three months. These results indicate that CBS activity is related to gestational age and that the cysteine need probably decreases with postnatal age. In addition, in vivo studies confirmed that cysteine synthesis was limited in preterm infants (74-76). Cysteine is indispensable for protein synthesis and is an important precursor for glutathione. If cysteine is a conditionally indispensable amino acid in preterm infants it could be the limiting factor for adequate glutathione production. Preterm birth and critical illness, including oxygen supplementation, might lead to higher glutathione requirement in these infants. If cysteine

concentration is inadequate, glutathione quantity might be insufficient to prevent oxidative stress.

SULFUR AMINO ACIDS REQUIREMENTS

The first minimal total sulfur amino acid requirements for adult humans (approximately 15 mg/(kg·d)) were reported in the fifties and was established by nitrogen balance (77). Recent studies have determined that the methionine requirements in school-aged children (78) are similar to young man (12.6 mg/(kg·d) (53). Initial data on methionine requirements for term infants fed adequate amounts of cystine date from 1964 and were based on nitrogen balance and weight gain rate (30). The methionine requirement in these infants ranged from 32 to 49 mg/(kg·d), so two to three fold higher than in older children and adults.

Cysteine is a dispensable amino acid in children and adults and therefore there is no need to determine cysteine requirements in these populations. If methionine is supplied in adequate amounts, cysteine availability will be guaranteed. On the other hand, since it is believed that cysteine is indispensable in infants born prematurely, the cysteine requirements for these infants are highly important.
Snyderman et al. (79) were the first to report cyst(e)ine requirements for preterm infants. They found in 2- to 4-month-old infants born prematurely that after a period of cyst(e)ine depletion intakes of 44 or 66 mg cyst(e)ine/(kg·d) did not restore nitrogen retention and weight gain to control values. They recommended a minimal intake of 85 mg cyst(e)ine/(kg·d). However, as they did not provide methionine intake, this recommendation might be overestimated in view of the risk of inadequate methionine intake. The cystine requirement for normal growth by full-term infants fed a soy-isolate formula has been reported to be less than 25 mg/100 kcal on a diet of 1.62 g of protein/100 kcal (80). Several years later, the same group determined the requirement for sulfur amino acids in two-month-old term infants fed 2.8 g of isolated soy protein/100 kcal and recommended a sulfur amino acids intake of 588 µmol/100kcal when methionine intake is 264 µmol/100kcal (39 mg/100 kcal).

The current sulfur amino acid requirements in infants and children are based on the factorial approach (81). Because no fetal cysteine accretion rate is available the factorial method is not applicable to estimate the cysteine requirements for preterm infants. To date, the cysteine requirement recommendation for preterm infants, i.e. 66-95 mg/(kg·d), a twofold higher than for term neonates and is based on the minimum and maximum amounts of each amino acid present in the amounts of human milk protein corresponding to the recommended minimum and maximum protein contents of 3.0 g/(120 kcal) and 4.3 g/(120 kcal), respectively (17). However, more direct estimates are warranted to fulfill the minimal cysteine need in these infants, particularly in regard with the presumable high turnover of cysteine due to oxidative stress. In this thesis the cysteine requirements for preterm infants are determined with use of the IAAO method.

INTESTINAL (SULFUR AMINO ACIDS) METABOLISM

The gastrointestinal tract has important metabolic functions during growth. For instance, it controls amino acid absorption, regulates the metabolic fates of amino acids in enterocytes and it also manages the availability of amino acids for peripheral tissues. Arginine supplementation in preterm infants seems to prevent necrotizing enterocolitis (82, 83) and enteral glutamine supplementation reduces the incidence of serious infections (84, 85). Thus, enteral feeding is obligatory for intestinal maturation, growth and development of other organs; so much effort should be directed towards understanding the nutritional needs of the intestine of preterm infants. Regarding the different metabolic fates of absorbed amino acid by enterocytes, it seems likely that the intestinal utilization of amino acids might influence the requirements for specific amino acids, for instance sulfur amino acids. Early studies by Mudd et al. (71) showed that CBS and cystathionine-lyase are active mainly in human liver tissue, and to a lesser extent, in extra hepatic tissues such as the small intestine. However, Stegink and den Besten (86) were the first to report in vivo evidence to implicate splanchnic tissues (intestine and liver) as a key site of methionine transsulfuration by demonstrating that plasma cysteine concentrations are significantly higher in human subjects fed a methionine-containing, cystine-free diet enterally versus intravenously (Figure 4). However, subsequent metabolic studies with stable isotopic tracers also have indirectly suggested that a substantial fraction of the dietary methionine undergoes transmethylation and remethylation during first-pass splanchnic metabolism in adult humans (87, 88). More recent studies show that whole-body methionine requirement and circulating homocysteine concentrations are significantly higher in piglets fed enterally than parenterally, demonstrating that splanchnic methionine metabolism is nutritionally significant (89, 90). If this phenomenon does occur in the preterm infant it will have tremendous effects on route of feeding in these infants.

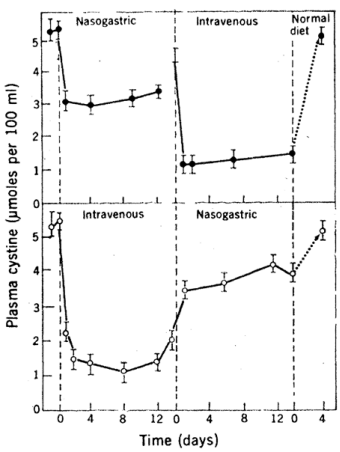

Figure 4. Plasma cystine concentrations (mean ± SD) in normal male subjects given parenteral alimentation solution. Solid circles are data for subjects fed first by nasogastric tube and then by intravenous infusion; the order of feeding routes was the reverse for other subjects (open circles). With permission, Science 1972 (86).

Methods to study intestinal metabolism

The intestine is unique in its simultaneous nutrient supply; amino acids are derived from both the diet (luminal site) and the systemic circulation (basolateral site). To study intestinal metabolism, one need to know the intestinal input and output and the latter is difficult to measure in humans. In piglets it is possible to study metabolism of the portal drained viscera (intestine, spleen, pancreas and stomach) with the use of a portal vein catheter to obtain portal blood. However, for ethical reasons it is not possible to study portal drained viscera metabolism in human subjects. Instead, the amino acid uptake by the splanchnic tissues (stomach, small intestine and liver) in first-pass, which is the amount of substrate used by the splanchnic tissues before it reaches the systemic circulation, can be determined with dual stable isotope infusions technique. This method is highly suitable to study metabolism in neonates. It requires an enteral infusion of a labeled amino acid of interest together with an intravenous infusion of the same amino acid but with a different labeling. The simultaneous infusion of these two tracers allows calculation of the splanchnic first-pass uptake of the amino acid under study. Enteral infusion of a labeled amino acid should result in the same plasma isotopic enrichment as the intravenous infused labeled amino acid if nothing is consumed in first pass. However, if the splanchnic bed utilizes a certain amount of the enterally administered tracer, less tracer enters the systemic circulation and eventually results in lower plasma enrichment than the intravenous administered tracer. This method assumes complete absorption and digestion of proteins. Incomplete digestion and absorption results in an overestimation of first pass utilization. Complete digestion may be assumed from the fact that less than 1% of enteral administered tracer is found in stools of adults (91) and that approximately 98% of the ingested human milk proteins are digested and absorbed in infants (92, 93). However, this does not concern free amino acid mixtures. In general, it appears that amino acid absorption from peptides is more rapid than from amino acid mixtures (94). Metges et al. (95) investigated the metabolic fate of dietary [1-^{13}C]leucine ingested together with either a mixture of free amino acids, intrinsically labeled protein or with unlabeled intact protein. They reported a higher leucine oxidation rate when [1-^{13}C]-leucine was ingested together with the L-amino acid mixture compared to the intrinsically labeled protein. To what extent the oxidation difference is due to a difference in absorption rate per se is not clear.

Intestinal utilization

Although the portal drained viscera (PDV) account for only 4-6% of the body weight, they utilize 10-20% of whole body energy expenditure and contribute for 20-50% to whole body protein turnover (96, 97). The high energy expenditure rate reflects the high metabolic activity of these tissues. Studies in neonatal piglets and humans showed that the PDV and splanchnic tissues extract large amounts of the dietary amino acids (98). As depicted in Table 2, splanchnic utilization rates among various dietary amino acids differ, and clearly both indispensable and dispensable amino acids are extensively extracted in first-pass. Studies in piglets have shown that nearly all threonine, glutamate and aspartate were extracted in first-pass metabolism (99, 100, 105) and only half of the dietary lysine was utilized by the PDV (97).

Comparable results have been reported in adult humans; splanchnic tissues retained 20-90% of the dietary intake of both indispensable and dispensable amino acids (Table 2). The first pass utilization of amino acids regulates the systemic availability, and thus the supply to other organs. In this view it is important to determine the splanchnic dietary amino acid extraction in preterm infants. Regrettably, only a few studies have determined the splanchnic uptake rates of some amino acids.

Table 2. First-pass splanchnic uptake in piglets, adults and preterm infants.

Amino acid	First pass splanchnic uptake of dietary intake (%)			Reference
	Piglet	Adult	Preterm infant	
Leucine	43	21	42-48	(101, 102)
Lysine	46	32	18	(97, 103)
Cysteine	< 20	44	-	(88, 104, 105)
Methionine	this thesis	33	this thesis	(88, 105, 106)
Phenylalanine	51	29	-	(101, 106)
Threonine	80		70	(99, 107)
Aspartate	95	-	this thesis	(100, 105)
Glutamate	92	96	this thesis	(100, 105, 108)
Glutamine	-	64	53	(109, 110)

Intestinal energy sources

Given the nutritionally significant proportion of the dietary amino acid intake that is extracted by the PDV and splanchnic tissues, it is of particular interest to determine the metabolic fate of these amino acids metabolized by these tissues. The possible metabolic fates of the sequestered amino acids in the gastrointestinal tissues in first-pass are: protein synthesis, intermediate metabolism, and irreversible oxidation to CO_2 and ammonia. Obviously, the intestine is an energy demanding organ. It is of particular interest which sources of energy the intestine utilizes to fulfill the high energy demand. Windmueller and Spaeth (111, 112) investigated the metabolic fate of amino acid in the intestine rats. They showed that luminal glutamate and aspartate and arterial glutamine were the major fuel sources for the enterocytes, and that glucose was a minor oxidative substrate. Subsequently, studies in neonatal pigs have revealed a similar pattern (97, 100). Glutamate appeared to be the major oxidative substrate and in a lesser extent glucose, lysine and leucine. In contrast, dietary threonine utilized in first-pass was not used for oxidative purposes but was predominantly used for intestinal mucosal proteins (99). Obviously, amino acids and glucose are the predominant fuel sources for the intestine in piglets.

It is of particular interest which fuels the splanchnic tissues of the preterm infants utilize. Previously, the splanchnic oxidation rates of glucose, lysine and threonine in preterm infants

have been investigated (103, 113). Approximately 80% of the utilized glucose in first-pass was oxidized and is therefore a major source of energy for the splanchnic tissues of preterm infants (113). Like in piglets, lysine (103) and threonine (107) were not used as an oxidative substrate in the splanchnic bed. Apparently, other substrates than glucose need to be used for energy generation in these tissues. It is likely that the same pattern seen in piglets resembles that of the preterm infant. However, no data are available on substrate oxidation in these infants, except for lysine and glucose. It needs to be evaluated in what extent the dietary dispensable amino acids glutamate and aspartate are used for fuel in the intestine in preterm infants in order to be able to meet the metabolic needs.

It is well recognized that enteral feeding has a major influence on short-term and long-term outcome in preterm infants. Nutrient requirements are higher in these infants than in term infants as a result of rapid growth and illness. It is therefore of great importance to provide adequate nutrition to meet their specific requirements to optimize growth and outcome. The studies in this thesis investigate cysteine requirements for preterm infants with different gestational ages at different postnatal ages were determined. Furthermore, we aimed to gain more insight into splanchnic sulfur amino acids metabolism in neonatal pigs and preterm infants. In view of the high intestinal energy expenditure, the role of dispensable amino acids, such as glutamate and aspartate, as oxidative substrates in splanchnic tissues of preterm infants was investigated to understand the pivotal metabolic role of these organs.

OUTLINE AND AIMS OF THE THESIS

The overall aims of the work presented in this thesis are:

1. To determine the cysteine requirements for preterm infants during the first three months of life.
2. To study sulfur amino acid metabolism in neonatal piglets and preterm infants.
3. To quantify the role of the intestine in sulfur amino acid metabolism in both neonatal piglets and humans.
4. To determine whether dispensable amino acids such as glutamate and aspartate contribute significantly to the high energy needs of the intestine.

The main hypotheses that will be tested are:

1. Cysteine is a indispensable amino acid for newborn preterm infants.
2. Cysteine requirements change with postnatal age.
3. The intestine is an important site for cysteine synthesis.
4. Methionine is extensively utilized in first pass metabolism.
5. The dispensable amino acids glutamate and aspartate are major energy sources for the splanchnic tissues in preterm infants.

Chapter 2 describes a study to validate the enteral infusion of labeled sodium bicarbonate. This was done in order to reduce the invasiveness of the method that is used to determine individual carbon dioxide production. This is usually determined by intravenous infusion of labeled sodium bicarbonate. We wanted to infuse the tracer enterally so no catheter was needed for the study.

Part 1. Cysteine requirements

The first part of this thesis describes studies to determine the cysteine requirement in (very) low birth weight preterm infants with different gestational ages and postnatal ages. In **Chapter 3**, the cysteine requirement is determined in infants born with gestational ages of 32-34 weeks, at the age of one month postnatally. **Chapter 4** describes the cysteine requirement in infants born with gestational ages of 26-29 weeks, determined at the age of four and eight weeks postnatally. The two studies provide information whether cysteine requirements are depending on gestational age and/or postnatal age.

Part 2. Sulfur amino acid metabolism

The second part describes the sulfur amino acid metabolism in piglets and preterm infants. **Chapter 5** describes the study investigating that the intestine, rather than the liver, is the major site of cysteine synthesis from methionine and serine. This study was performed in neonatal pigs. **Chapter 6** describes a study to determine the splanchnic first-pass methionine uptake in fully enterally fed preterm infants and links with chapter 5. The conversion of methionine and serine into cysteine was also investigated in these infants.

Part 3. Splanchnic metabolism of dispensable amino acids

This part describes the splanchnic metabolism of the dispensable amino acids glutamate and aspartate in preterm infants. **Chapter 7** describes the splanchnic first-pass metabolism of dietary glutamate in preterm infants. In **Chapter 8** the splanchnic first-pass metabolism of aspartate is presented.

Part 4. Discussion and Summary

In the general discussion, **Chapter 9**, the results obtained in this thesis are discussed on the basis of relevant reviewed literature. Also, suggestions of future nutritional research in preterm infants are proposed. Finally, the results of the studies are summarized in **Chapter 10**.

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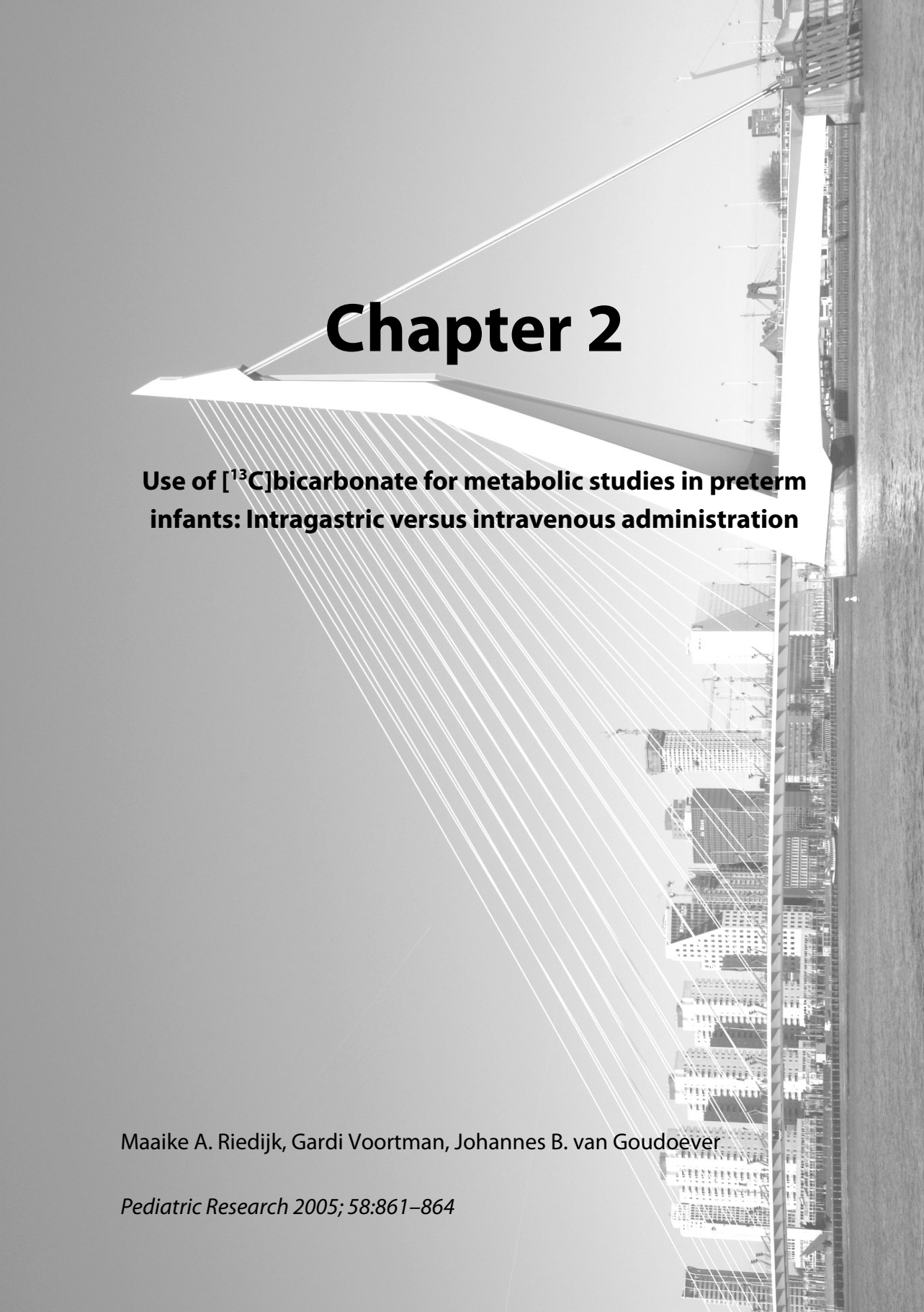

Chapter 2

Use of [^{13}C]bicarbonate for metabolic studies in preterm infants: Intragastric versus intravenous administration

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ABSTRACT

The metabolic fate of substrates in humans can be examined using stable isotopes, one of which, [^{13}C]bicarbonate, may serve to estimate CO_2 production rate. In view of minimizing the burden of metabolic studies for preterm infants, the authors determined whether intragastric and intravenous infusions of [^{13}C]bicarbonate would achieve the same $^{13}CO_2$ enrichment in expired air during steady state. A second aim of this study was to determine the minimum time required to reach steady state during intragastric infusion.

Ten preterm infants received a primed continuous [^{13}C]bicarbonate infusion intragastrically, followed by an intravenous infusion the next day. Breath samples were obtained every 30 min by the direct sampling method. $^{13}CO_2$ isotopic enrichment, expressed as atom percent excess, was measured by isotopic ratio mass spectrometry. Two-tailed *t* tests were used to detect statistically significant differences between the infusion routes.

The isotopic enrichment at plateau did not differ between intragastric and intravenous infusion. A steady state of $^{13}CO_2$ enrichment was achieved after 60 min of intravenous infusion and after 120 min of intragastric infusion.

In conclusion, intragastric infusion of [^{13}C]bicarbonate may serve to estimate whole-body CO_2 production rate in preterm infants. To reach $^{13}CO_2$ steady state, a minimum of 120 min of bicarbonate administration is required.

INTRODUCTION

The past two decades have seen increased use of stable isotopes to study amino acid metabolism in humans. These isotopic tracer techniques have greatly enhanced our understanding of nutrient daily requirements and metabolism (1).

For determining the oxidation rates of specifically labeled substrates such as amino acids or glucose, we need to quantify substrate oxidation in each individual by measuring the $^{13}CO_2$ production rate during intravenous (IV) infusion of labeled bicarbonate (2). The production of $^{13}CO_2$ is made up of total CO_2 production rate and $^{13}CO_2$ enrichment in expired breath. Although total CO_2 production rate is traditionally assessed by indirect calorimetry, $^{13}CO_2$ enrichment is measured by isotopic ratio mass spectrometry. A certain amount of CO_2, and thus $^{13}CO_2$ as well, is retained in the body. Because this amount is related to caloric intake, a correction factor is necessary to calculate substrate oxidation rates (3). A method that makes correction factors and indirect calorimetry superfluous is the infusion of $NaH^{13}CO_3$ before the labeled substrate infusion (4).

Kien et al. (5) published a study where they compared intragastric (IG) infusion of [^{13}C]-bicarbonate to indirect calorimetry by the use of a correction factor. This study showed the validation of the use of dilution stable tracer technique to estimate CO_2 production. However, those authors did not compare the $^{13}CO_2$ enrichment during IV infusion with IG infusion of [^{13}C]bicarbonate.

The general purpose of this study was to determine whether in preterm infants IG infusion of $NaH^{13}CO_3$ yields the same enrichment as IV infusion at steady state. To this aim, we compared $^{13}CO_2$ enrichment in expired breath during IG and IV infusion of labeled bicarbonate at plateau. In addition, we quantified the minimal tracer infusion time required to establish steady state during IG infusion.

We hypothesized that $^{13}CO_2$ enrichment at steady state would not differ between IG administration and IV administration of [^{13}C]bicarbonate.

METHODS

Subjects

We studied 10 preterm infants (8 male, 2 female) admitted to the Neonatal Intensive Care Unit of the Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands. Their mean gestational age was 27 wk (range: 26-30 wk, SD $\pm$ 1.3 wk), and they were free of gastrointestinal diseases and were clinically stable during the 2-day study. Five of them needed artificial ventilation and five breathed spontaneously with O_2 supplementation by nasal prong (n=5). Eight infants tolerated full enteral feeding and two infants received partial enteral and partial parenteral feeding. For all neonates, the feeding regimen was the same on both study days. All infants were fed through a nasogastric feeding tube, because this is a standard procedure in our unit. The study protocol was approved by the Erasmus MC Institutional Review Board, and written and informed consent was obtained from both

parents of all neonates.

Tracer Protocol

For the purpose of validating this route of labeled sodium bicarbonate the study was designed as a randomized, crossover study. The 10 infants received a primed (10 µmol/(kg·min)) continuous (10 µmol/(kg·h)) infusion of [^{13}C]bicarbonate (sterile pyrogen free, 99% atom percent excess (APE); Cambridge Isotopes, Woburn, MA). The study was set up as a true crossover design: in five infants the IV infusion was started for 6 hours on the first day, followed by the IG infusion on the second day. The other five infants received the IG infusion the first day and the IV infusion the second day. One hour before start of the study, the usual hourly feeding regimen was changed into continuous dripfeeding. Enterally infused tracer was mixed with the milk (either fortified or non fortified breast milk, or preterm infant formula; Nenatal, Nutricia Nederland B.V, Zoetermeer, the Netherlands) and infused continuously via the nasogastric tube.

Breath samples were obtained by use of the direct sampling method described by van der Schoor et al. (6). Briefly, in mechanically ventilated neonates, a syringe was connected to the ventilator tubing, and breath was taken slowly during expiration with a total volume of 15 mL. When infants were breathing spontaneously, a 6F gastric tube (6 Ch Argyle; Cherwood Medical, Tullamore, Ireland) was placed 1 to 1.5 cm into the nasopharynx and end-tidal breath was taken slowly with a syringe connected at the end. Collected air was transferred into 10 mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and stored at room temperature until analysis.

Baseline samples were obtained 15 and 5 min before tracer infusion was started. During the experiment, duplicate ^{13}C-enriched breath samples were collected every 30 min and every 15 min during the last 45 min of tracer infusion.

Analytical Methods

$^{13}CO_2$ isotopic enrichment in expired air was measured by isotope ratio mass spectrometry (ABCA; Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands) and expressed as APE above baseline. The APE was plotted relative to time. Steady state was defined as three or more consecutive points with a slope not different from zero. Estimated body CO_2 production (mmol/(kg×h) was calculated for each infant with the following equation (7):

$$\text{Estimated body } CO_2 \text{ production} = [\text{ IE infusate/ IE breath bicarbonate -1 }] \times \text{tracer infusion rate} \times 1000$$

where IE infusate is the ^{13}C enrichment of the tracer (APE), IE breath bicarbonate is the ^{13}C enrichment in the expired air (APE), and tracer infusion rate is the rate of [^{13}C]bicarbonate infusion (mmol/(kg·h)).

Statistical analysis

Descriptive data are expressed as mean ± SD. To define the slope of the curve of the two different methods a repeated measurements linear model was used. Steady state was

achieved when the linear factor of the slope was found to be not significantly different from zero ($p > 0.05$) (8). Whole-body CO_2 production and baseline enrichments between the two methods were analyzed by paired *t* tests.

Differences in steady state between IG and IV administration were also analyzed by paired *t* tests. Statistical significance was defined as $p < 0.05$. Pitman's test (9) was used to test the null hypothesis if the variance of two-paired measurements (IG and IV infusion) were the same. To detect significant differences between the two-paired measurements, a paired *t* test could be performed. Pearson's correlation coefficient was performed to show correlation between IG and IV. The analysis of Bland and Altman (10) was performed to show accuracy between the two different infusions. All statistical analyses were performed by the use SPSS (version 11.0; SPSS Inc, Chicago, IL).

RESULTS

The clinical characteristics of the subjects are given in Table 1. The mean study weight of the infants was 1.18 $\pm$ 0.32 kg. The postnatal age at the start of the study was 28 $\pm$ 20 d. Their energy intakes did not differ between both study days ($p = 0.75$). The mean ^{13}C enrichments, expressed as atom percent (AP), in breath CO_2 from time point t=60 to t=360 min are shown in Figure 1. All neonates achieved isotopic steady state in both administration routes. Baseline enrichments did not differ between IG and IV infusion (1.0875 AP $\pm$ 0.0022 *versus* 1.0869 AP $\pm$ 0.0338, $p = 0.29$).

The mean APE at plateau ($t_{120\text{-}360}$) during IG infusion was 0.0365 $\pm$ 0.0055; during IV infusion it was 0.0371 $\pm$ 0.0067. IG enrichment was slightly lower, though not significantly, than IV enrichment ($p = 0.59$).

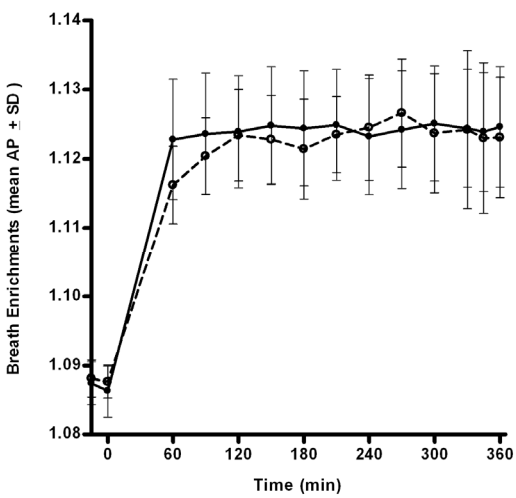

Figure 1. Breath $^{13}CO_2$ enrichments of 10 infants, expressed as mean AP $\pm$ SD. IV (·) *versus* IG (º) infusion of [^{13}C]-bicarbonate. At plateau (t_{120}-t_{360}) ^{13}C enrichment IV is not significantly different from IG ($p = 0.59$).

Table 1. Clinical characteristics of 10 studied infants.

	Sex	Gestational age (wk)	Birth weight (kg)	CRIB score	Respiratory support	Feeding Regimen e/p	Energy intake kcal/kg/d IV	Energy intake kcal/kg/day IG
1	M	26	0.97	-	np	e	154	153
2	M	26	0.70	13	np	e	128	118
3	F	27	1.13	2	v	e	102	104
4	M	26	0.79	4	v	e	121	123
5	M	27	1.05	2	v	e	98	98
6	F	29	0.81	4	np	e	131	134
7	M	27	1.18	1	np	e	134	131
8	M	27	0.67	9	V	e	109	109
9	M	30	1.17	1	np	e+p	112	112
10	M	27	0.88	4	V	e+p	95	98
Mean		27	0.93	4			118	118
SD		1	0.19	4			19	18

Key: e = enteral intake; p = parenteral intake; v = ventilation, np = nasal prong.

The pitman's tests (9) showed no significant difference between variance in IV and IG infusion (p = 0.308), and Pearson's correlation coefficient was 0.359. Agreement between the two different routes of administration was determined by the analysis of Bland and Altman (10). Figure 2 shows on the x axis the average of IV plateau and IG plateau (n=10), whereas the y axis shows the difference between the two measurements (n=10). The mean difference is 0.0006 APE. Note that all measurements lie between the range of the mean difference + 2SD (0.0076 APE) and the mean difference – 2SD (-0.0064 APE). The 95% CI of the mean difference is –0.0019 to 0.0031 APE. Therefore, from 120 min onward, there was no statistically significant difference in CO_2 enrichment in expired air between IV or IG infusion, nor did we find a sequence effect (no significant difference in $^{13}CO_2$ between infants who received $NaH^{13}CO_3$ IV the first day or those who received $NaH^{13}CO_3$ IG the first day).

The estimated CO_2 production did not differ between the IG (27.68 $\pm$ 5.38 mmol/(kg·h)) and IV (27.67 $\pm$ 5.64 mmol/(kg·h)) infusions (p = 0.99).

Steady state was achieved from 60 min onward when the tracer was infused IV and from 120 min onward when infused IG.

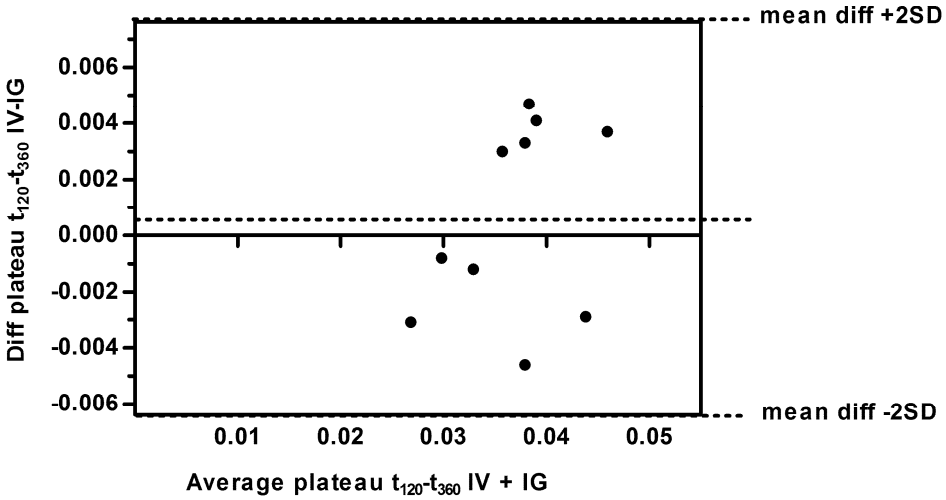

Figure 2. Analysis of Bland and Altman showing the dfference between IG and IV enrichments of $^{13}CO_2$ in breath of 10 infants. The mean difference is 0.0006 APE (dotted line). All measurements are within two standard deviations: + 2 SD (0.0076 APE) and – 2 SD (-0.0064 APE). The 95% CI of the mean difference is -0.0019 to 0.0031 APE.

DISCUSSION

The main purpose of this study was to validate the use of IG administration of [^{13}C]-bicarbonate compared with IV administration for metabolic oxidation studies in preterm infants. Clinical studies in addition to experimental research are of great value in elucidating metabolism and nutrition in preterm infants. Information about amino acid metabolism and protein synthesis and oxidation is needed to provide these infants with optimal nutrition and consequently improved growth and survival.

A principal goal of many tracer kinetic experiments is to determine the oxidation rate of the tracer substance by the appearance in breath of labeled C originating from the tracer (11). The gold standard for determining whole-body CO_2 production is by indirect calorimetry (3). An alternative method is a primed continuous IV infusion of $NaH^{13}CO_3$. We found the estimated body CO_2 production (27.67 ± 5.64 mmol/(kg·h)) to be similar to that previously described (0.725 $\pm$ 0.021 mol/(kg·d)) (12). Also, others have shown that $NaH^{13}CO_3$ can be adequately used as a method of determining CO_2 production rate (4, 5, 13). The infusion of labeled bicarbonate before a ^{13}C-labeled substrate carries the advantage that no correction factor is needed to calculate substrate oxidation. In addition, IG infusion of the tracer reduces the invasiveness of metabolic studies. Finally, in studying the metabolic fate of an enteral substrate it is preferable to administer the tracer enterally as well.

Hoerr et al. (11) studied in adults the effects of IG and IV infusion of labeled bicarbonate on recovery of ^{13}C in breath and concluded that administration route did not affect recovery. When it is considered that placing an intravenous catheter in preterm infants is highly invasive, it is very important to search for methods minimizing discomfort.

To achieve steady state during IG administration, tracer infusion should last at least 120 min. Sample collection is accomplished during steady state. Consequently, breath samples should be obtained from 120 min onward. To prevent intrasubject variation, at least four breath samples should be obtained at 10-min intervals, thus between 120 and 160 min of infusion.

We need to emphasize the small sample size of this study. However, we presented a 95% CI (-0.0019 to 0.0031 APE) of the mean difference to obtain an impression of a type II error. We considered a difference of < 10% between IG plateau and IV plateau acceptable. We calculated the difference of the minimal (-5%) and maximal (8%) of the 95% CI limit of the average plateau of IG and IV (0.0368 APE). As we assumed, the plateau of IV and IG infusion can vary from –5% to 8% in the general population.

Additionally, we wish to stress that in metabolic studies in parenterally fed infants, [^{13}C]-bicarbonate should preferably be administrated IV.

In conclusion, our findings are consistent with the absence of significant differences in $^{13}CO_2$ enrichment between IG and IV infusion after 120 min of infusion, and therefore it would be valid to infuse [^{13}C]bicarbonate IG for the determination of whole-body CO_2 production rate in preterm infants.

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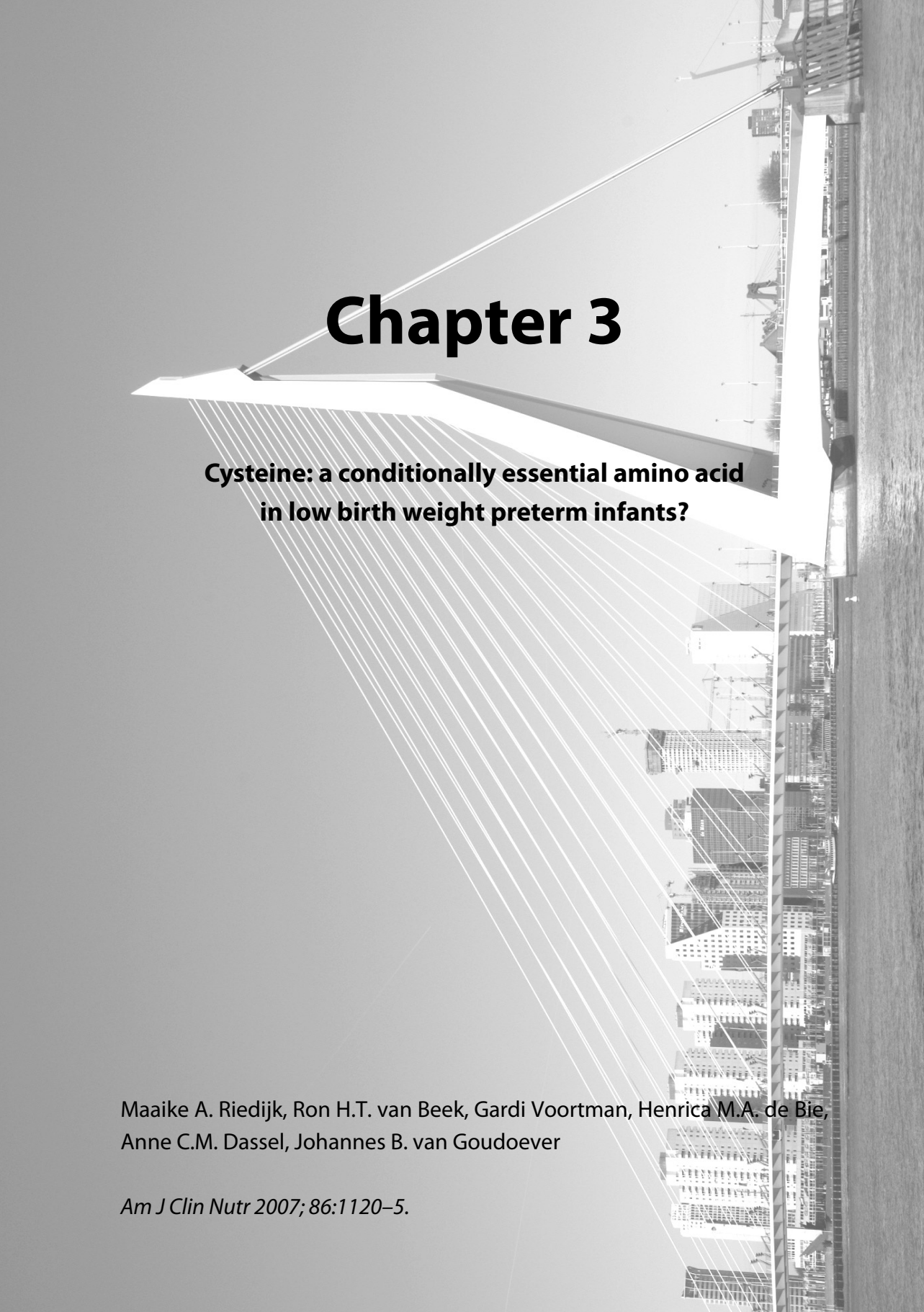
Cysteine requirements



Chapter 3

Cysteine: a conditionally essential amino acid in low birth weight preterm infants?

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ABSTRACT

Cyst(e)ine can be synthesized de novo from methionine and serine and is, therefore, a nonessential amino acid in human adults. Several studies have suggested that cyst(e)ine might be a conditionally essential amino acid in preterm infants because of biochemical immaturity. No data are available on cyst(e)ine requirements in low-birth-weight (LBW) preterm infants.

The aim was to determine cyst(e)ine requirements in LBW infants with gestational ages from 32 to 34 wk, measured 1 month after birth with the use of indicator amino acid oxidation technique.

LBW infants were randomly assigned to 1 or 2 of the 5 formulas containing graded cystine concentrations (11, 22, 32, 43, 65 mg cyst(e)ine/100 mL) and generous amounts of methionine. After 24-h adaptation, cyst(e)ine requirement was determined by $^{13}CO_2$ release from [1-^{13}C]phenylalanine in expired breath. $^{13}CO_2$ enrichment was measured by isotopic ratio mass spectrometry.

Cyst(e)ine requirement was determined in 25 LBW infants with a mean (± SD) gestational age of 33 ± 1 wk and birth weight of 1.78 ± 0.32 kg. Fractional oxidation of [1-^{13}C]-phenylalanine did not differ between the 5 groups.

There is no evidence for limited endogenous cyst(e)ine synthesis in 4-wk-old LBW preterm infants born at gestational ages from 32 to 34 wk. It is safe to conclude that the cyst(e)ine requirement is < 18 mg/(kg·d) providing generous amounts of methionine and that cyst(e)ine is probably not a conditionally essential amino acid in fully enterally fed LBW preterm infants born at 32-34 wk.

INTRODUCTION

Note: 'Cyst(e)ine' is used throughout to designate any undefined combination of cysteine and cystine; the term 'cysteine' and 'cystine' are used to designate the specific amino acids.
Cyst(e)ine is a sulfur-containing amino acid that is not essential in humans. The human body synthesizes cyst(e)ine *de novo* from methionine, the only essential sulfur-containing amino acid, and serine. Cyst(e)ine has several important metabolic functions. First, like all other amino acids it is involved in growth and protein synthesis. Furthermore, it is a precursor for the tripeptide glutathione, an important intracellular antioxidant. Then, it is also a precursor for the production of taurine, another anti-oxidant, and sulfate.
Some nonessential amino acids are classified as conditionally essential. These amino acids may become temporarily essential when synthesis during rapid growth or critical illness is insufficient. Cyst(e)ine is believed to be conditionally essential in preterm infants because of biochemical immaturity of the enzyme cystathionase (EC 4.4.1.1) that is involved in cyst(e)ine synthesis (1-3). It is, therefore, important to know the exact cyst(e)ine requirement of preterm infants and also in view of the higher amino acid requirement as a result of rapid growth and development.
Different methods are used to estimate individual amino acid requirements, e.g. the nitrogen balance method, growth rate, plasma amino acid patterns, and the factorial approach. The first reports on amino acid requirements in neonates were based on nitrogen balance and weight gain rate (4, 5). Nitrogen balance has several limitations (6), for instance it requires 7-10 d to adapt to the test diet (7). Because present-day practice will not allow neonates to be maintained on either deficient or excess intakes of amino acids for a minimum of 7 d, no recent requirement studies have used this method in preterm infants.
At present, the factorial approach is used to define amino acid requirements in infants (8). This model is based on fetal protein accretion during normal intrauterine development. Accretion data are derived from body carcass analysis of stillborn preterm infants, some born > 100 years ago (9, 10). Not all data are suitable as reference material; gestational age and cause of death were not accurately obtained (11), and in view of this study, cysteine content of the body was not determined. The current recommendation for cyst(e)ine requirement for preterm infants, ie, 66-95 mg/(kg·d), is based on the minimum and maximum amounts of each amino acid present in the amounts of human milk protein corresponding to the recommended minimum and maximum protein contents of 3.0 g/120 kcal and 4.3 g/120 kcal, respectively (8).
Because methionine is the precursor of cyst(e)ine, the methionine intake needs to be taken into account as well. The current estimated methionine requirement for preterm infants is 48 -69 mg/(kg·d) (8). To ensure an adequate methionine intake, the study formula supplied an amount of 70 mg methionine/(kg·d). Shoveller et al. (12) determined the total sulfur amino acid requirement in enterally fed neonatal piglets when no dietary cysteine was provided. The extrapolation from this piglet study results in a methionine intake of 84 mg/(kg·d) for the human neonate, which is slightly above the current recommendations (8).
The objective of the present study was to use indicator amino acid oxidation (IAAO) with

[1-^{13}C]phenylalanine to estimate cyst(e)ine requirements in fully enterally fed low-birth-weight (LBW) infants supplied with an adequate methionine intake. We hypothesized that cyst(e)ine is a conditionally essential amino acid for these infants.

SUBJECTS AND METHODS

Subjects

Subjects eligible for the study were LBW infants with gestational age (GA) between 32 and 34 wk, admitted to the neonatal department of the Amphia Hospital, Breda; Vlietland Hospital, Vlaardingen; or Deventer Hospital, Deventer (all: the Netherlands). The infants needed to be clinically stable during the study, and those with any congenital or gastrointestinal disease were excluded. All tolerated full enteral feeding and were partly fed through a nasogastric feeding tube and partly bottle-fed. The study protocol was approved by The Central Committee on Research Involving Human Subjects (CCMO) and the Erasmus MC Institutional Review Board. Written and informed consent was obtained from both parents of each subject.

Study formula

We used 5 study formulas containing graded cyst(e)ine concentrations: 11, 22, 32, 43 and 65 mg cystine/100 mL (Xcys/Neocate, Nutricia Nederland BV, Zoetermeer, the Netherlands/SHS International, Liverpool, United Kingdom). Infants were enrolled in the study when they tolerated full enteral feeding (>150 mL/(kg·d)). Except for cyst(e)ine concentration, the 5 formulas did not differ as to amino acid composition (Table 1), and consequently the nitrogen content increased slightly from 0.37 mg nitrogen/100 mL (formula 1) to 0.38 mg nitrogen/100 mL (formula 5). The graded cyst(e)ine concentrations of the study formula were based on the current estimated cyst(e)ine requirement for preterm infants (8), which is 66-95 mg/(kg·d), and is based on the minimum and maximum amounts of each amino acid present in the amounts of human milk protein corresponding to the recommended minimum and maximum protein contents of 3.0 g/120 kcal and 4.3 g/120 kcal, respectively. We decided to test 3 cyst(e)ine intakes above and 2 cyst(e)ine intakes below the estimated cyst(e)ine requirements for formula-fed preterm infants. Methionine content was similar in all formulas and was supplied generously.

Study design and tracer protocol

Cyst(e)ine requirement was measured approximately 1 month after birth (range: 35-37 wk postmenstrual age). Infants were randomly assigned to at least one of the study formulas. The study diet was initiated 24 h before start of the oxidation study, and the dietary intake was not changed until the tracer protocol was finished. All subjects received approximately 170 mL formula/(kg·d) to ensure that all essential amino acids other than cyst(e)ine were in excess (Table 1) and, therefore, were not limiting for protein synthesis. Before the introduction of the study formula, almost all infants received their mothers' (expressed)

Table 1. Amino acid profiles of the study formulas.

Amino acid (mg/170 mL)	Formula 1	Formula 2	Formula 3	Formula 4	Formula 5
L-alanine	166	166	166	166	166
L-arginine	292	292	292	292	292
L-aspartic acid	274	274	274	274	274
L-cystine	18	36	54	72	109
L-Glycine	259	259	259	259	259
L-Histidine	168	168	168	168	168
L-Isoleucine	266	266	266	266	266
L-Leucine	435	435	435	435	435
L-Lysine	300	300	300	300	300
L-Methionine	70	70	70	70	70
L-Proline	313	313	313	313	313
L-Phenylalanine	197	197	197	197	197
L-Serine	194	194	194	194	194
L-Threonine	215	215	215	215	215
L-Tryptophan	88	88	88	88	88
L-Tyrosine	197	197	197	197	197
L-Valine	282	282	282	282	282
L-Asparagine	0	0	0	0	0
L-Citrulline	0	0	0	0	0
L-Carnitine	3	3	3	3	3
L-Taurine	8	8	8	8	8
L-Glutamine/ glutamate	371	371	371	371	371
Total (g/170 mL)	4.12	4.13	4.14	4.17	4.21

breast milk and only a few infants received standard preterm formula (Nenatal; Nutricia, Zoetermeer, the Netherlands). The cyst(e)ine concentration of human milk varies widely, but the preterm formula provided 35 mg cyst(e)ine/100 mL.

The IAAO technique uses as indicator a labeled essential amino acid that is different from the test amino acid. The indicator is independent of the different intake amounts of the test amino acid. If the test amino acid is deficient in the diet, this will limit overall protein synthesis and all other essential amino acids will be oxidized. Increasing the dietary intake of the test amino acid will linearly decrease oxidation of the indicator until requirement of the test amino acid is met. We chose [1-^{13}C]phenylalanine as the indicator (13).

After 24-h adaptation, subjects received a primed (10 µmol/kg) continuous (10 µmol/(kg·h)) enteral infusion of [^{13}C]bicarbonate (sterile pyrogen free, 99% atom percent excess (APE); Cambridge Isotopes, Woburn, MA) for 2.5 h to quantify individual carbon dioxide production. We infused the tracer enterally to minimize invasiveness, which has been validated by our group (14). The labeled sodium bicarbonate infusion was directly followed by a primed (30 µmol/kg) continuous (30 µmol/(kg·h)) enteral infusion of [1-^{13}C]phenylalanine (93% APE; Cambridge Isotopes) for 5 h. One hour before start of the oxidation study the feeding regimen was changed into continuous drip-feeding. Enterally infused tracer was mixed with the study formula and infused continuously by an infusion pump through the nasogastric tube. All infants were breathing spontaneously and 14 infants needed supplemental oxygen by a nasal prong.

Breath samples were obtained with the use of the direct sampling method described by van der Schoor et al. (15). In brief, a 6F gastric tube (6 Ch Argyle; Cherwood Medical, Tullamore, Ireland) was inserted 1 to 1.5 cm into the nasopharynx, and end-tidal breath was taken slowly with a syringe connected at the end. Collected air was transferred into 10-mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and stored at room temperature until analysis. Baseline samples were obtained 15 and 5 min before starting tracer infusion. Duplicate ^{13}C-enriched breath samples were collected every 30 min and every 15 min during the last 45 min of the tracer infusion.

Analytical methods and calculations

$^{13}CO_2$ isotopic enrichment in expired air was measured by isotope ratio mass spectrometry (ABCA; Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands) and expressed as APE above baseline (15). APE was plotted relative to time.

It is necessary to determine the individual carbon dioxide production to determine the rate of substrate oxidation. In general, the rate of oxidation is calculated by multiplying the isotopic enrichment of carbon dioxide in breath by the total rate of carbon dioxide excreted, the latter needs to be determined by indirect calorimetry to estimate the rate of carbon dioxide production. Alternatively, body carbon dioxide production can be determined by quantifying the excretion of $^{13}CO_2$ in expired air during [^{13}C]sodium bicarbonate infusion, which avoids the quantification of total expired air (15).

Estimated body carbon dioxide production (in mmol/(kg·h) was calculated as described previously (14). The rate of fractional [1-^{13}C]phenylalanine oxidation was calculated as

follows:

$$\text{Fractional phenylalanine oxidation (\%)} = [IE_{PHE} \times i_B]/[\, i_{PHE} \times IE_B] \times 100 \quad (1)$$

where IE_{PHE} is the ^{13}C isotopic enrichment in expired air during [1-^{13}C]phenylalanine infusion (in APE), i_B is the infusion rate of [^{13}C]bicarbonate (in µmol/(kg·h)), i_{PHE} is the infusion rate of [1-^{13}C]phenylalanine (in µmol/(kg·h)) and IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]bicarbonate infusion (16).

Statistical analysis

Descriptive data are expressed as mean ± SD. Steady state of $^{13}CO_2$ in expired breath during the [1-^{13}C]phenylalanine was achieved when the linear factor of the slope was found not to be significantly different from zero ($p \geq 0.05$). Cyst(e)ine requirement was determined with the IAAO method. The indicator oxidation rate is plotted against the varying dietary intakes of cyst(e)ine. The inflection or breakpoint in the indicator oxidation rate represents the physiological requirement of cyst(e)ine (17). Data were analyzed with the use of mixed model analysis of variance in SPSS software (version 14.0; SPSS Inc, Chicago, IL), while encoding the patients who participated twice with the same number. Repeated measures analysis of variance was performed on primary and derived variables to assess the effects of dietary intake and of subjects. Regression analysis was performed to analyze oxidation rates. Power calculation revealed that, assuming 5 formula groups with a group variance of 16, an intergroup variance of 5.5 and a power of 80%, a breakpoint should be detected with 6 subjects per group. Statistical significance was assumed at 5% level of significance ($p \leq 0.05$).

RESULTS

We included 25 LBW infants (12 boys, 13 girls) born at mean (± SD) GA of 33 ± 1 wk (range: 32-34 wk). They were studied at mean postmenstrual age 36 ± 1 wk (range: 35-38 wk), ie, approximately at postnatal age 1 month. Subject characteristics are depicted in Table 2. Five infants participated twice and received 2 different study formulas. Aiming at 6 measurements per formula, we performed a total of 30 labeled phenylalanine oxidation rate measurements in these 25 infants.

GA, study age, birth weight, and study weight did not differ between the 5 formula groups as shown in Table 2. In addition, the total enteral intake did not differ between the 5 formulas ($p = 0.07$). Although the nitrogen content of study formula 5 was somewhat higher, the nitrogen intake did not differ significantly between the formula groups ($p = 0.25$).

To detect any differences between the 5 groups receiving the different formulas we corrected for sex, study age, and study weight. The baseline ^{13}C enrichment in expired breath did not differ between the 5 formula groups (-17.24 ± 0.56, -16.77 ± 0.62, -17.95 ± 1.38, -17.87 ± 1.01 and –17.58 ± 1.55 Pee Dee Belemnite respectively; $p = 0.67$) after correction

Table 2. Patient characteristics.

Formula	Subjects n	GA wk	SA wk	BW kg	SW kg	Enteral intake mL/(kg·d)	Cyst(e)ine intake mg/(kg·d)	SAA intake mg/(kg·d)
1	6	33 ± 1	36 ± 1	1.88 ± 0.28	2.26 ± 0.24	170 ± 6	18 ± 1	89 ± 3
2	6	32 ± 1	35 ± 0	1.81 ± 0.36	2.10 ± 0.38	167 ± 1	36 ± 0	105 ± 1
3	6	33 ± 1	36 ± 1	1.81 ± 0.38	2.17 ± 0.38	167 ± 2	54 ± 1	123 ± 1
4	6	33 ± 1	36 ± 0	1.81 ± 0.24	2.17 ± 0.27	163 ± 5	70 ± 2	138 ± 4
5	6	32 ± 1	36 ± 1	1.58 ± 0.38	2.04 ± 0.31	166 ± 3	108 ± 2	177 ± 4
Mean	30	33	36	1.78	2.15	167		
SD		1	1	0.32	0.31	5		

GA, gestational age; SA, study age; BW, birth weight; SW, study weight; SAA, sulfur amino acids. No significant differences were detected in GA ($p = 0.20$), SA ($p = 0.38$), BW ($p = 0.55$), SW ($p = 0.86$) and enteral intake ($p = 0.07$) between the 5 formula groups. Rates expressed as mean ± SD

for birth weight and study weight (Table 3). Each subject reached a plateau during both [^{13}C]bicarbonate and [1-^{13}C]phenylalanine tracer infusions. As an illustration, the ^{13}C enrichments in expired breath during the infusion of [1-^{13}C]phenylalanine of 6 subjects receiving 54 mg cyst(e)ine/(kg·d)) are shown in Figure 1.

Table 3. Whole-body carbon dioxide production and fractional [^{13}C]phenylalanine (phe) oxidation rates of 5 different cyst(e)ine intakes.

Cyst(e)ine intake	Baseline $^{13}CO_2$	$^{13}CO_2$ [^{13}C]bicarb	CO_2 production	$^{13}CO_2$ [1-^{13}C]phe	Fractional oxidation of [1-^{13}C]phe
mg/(kg·d)	PDB	APE	mmol/(kg·d)	APE	%
18 (n=6)	-17.24 ± 0.56	0.0331 ± 0.0046	30.78 ± 5.31	0.0209 ± 0.0080	20.32 ± 5.26
36 (n=6)	-16.77 ± 0.62	0.0353 ± 0.0030	28.19 ± 2.61	0.0200 ± 0.0058	18.73 ± 5.03
54 (n=6)	-17.95 ± 1.38	0.0339 ± 0.0035	29.83 ± 3.08	0.0206 ± 0.0043	20.33 ± 4.15
72 (n=6)	-17.87 ± 1.01	0.0329 ± 0.0023	30.20 ± 1.97	0.0210 ± 0.0049	21.48 ± 5.70
109 (n=6)	-17.58 ± 1.55	0.0344 ± 0.0028	29.24 ± 2.22	0.0197 ± 0.0054	19.07 ± 4.93

Data expressed as mean ± SD. $^{13}CO_2$, enrichment of ^{13}C in expired air; PDB, Pee Dee Belemnite; bicarb, sodium bicarbonate; APE, atom percent excess; phe, phenylalanine. No significant differences were detected in baseline (p=0.67), CO_2 production (p=0.61) and fractional oxidation of labeled phenylalanine (p=0.73) between the five formula groups.

The mean fractional [1-^{13}C]phenylalanine oxidation did not differ between the groups (p = 0.73). Regression of the data did not show a linear decrease in indicator oxidation rate and, consequently a breakpoint is missing (Figure 2). A trend in the formula could not be detected (p = 0.90). This implies that the cyst(e)ine requirement under these circumstances is already met at an intake of 18 mg cystine/(kg·d). At the intakes of 18-109 mg/(kg·d), cyst(e)ine is not the limiting amino acid for protein synthesis and is therefore not deficient in the diet.

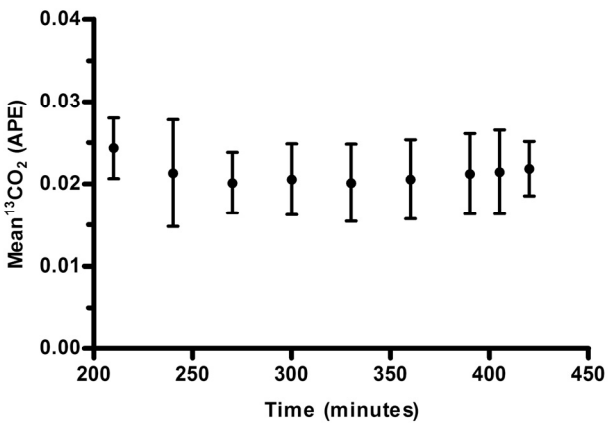

Figure 1. Mean ^{13}C enrichments in expired breath during enteral [1-^{13}C]-phenylalanine infusion in six infants receiving 54 mg cyst(e)ine/(kg·d). Data are expressed as mean ± SD. APE, atom percent excess.

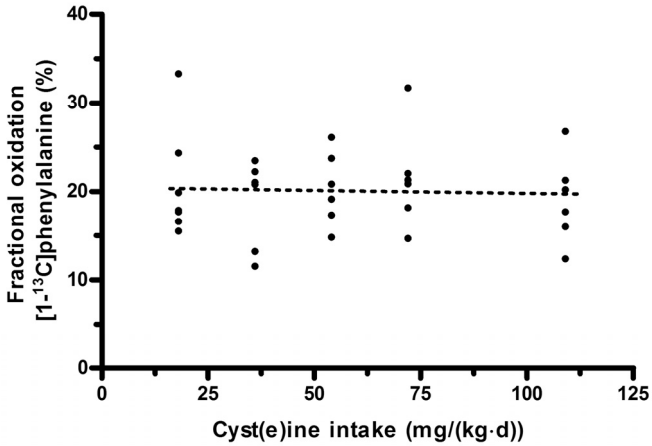

Figure 2. Fractional [1-^{13}C]phenylalanine oxidation plotted against increasing cyst(e)ine intake. Data are expressed as mean ± SD. No differences could be detected between the fractional oxidation of [1-^{13}C]phenylalanine of the 5 different cyst(e)ine intakes (n = 6 per intake; $p = 0.73$).

DISCUSSION

To our knowledge this the first study to determine the exact cyst(e)ine requirement in LBW infants. With the use of the IAAO method, we found it to be < 18 mg/(kg·d) 1 month after birth, which suggests that cyst(e)ine is not a conditionally essential amino acid in these infants at this age who are receiving generous amounts of methionine. The IAAO method is based on the assumption that the partition of any essential amino acid between oxidation and protein synthesis is sensitive to the amount of the most limiting amino acid in the diet (17, 18). Thus, a limitation of this method is the necessity of providing all amino acid in excess, except for the one under study. Accordingly, insufficient amounts of essential amino acids could have resulted in another essential amino acid than cystine being limiting for protein synthesis. However, we do not believe this is the case in this study, seeing that the fractional [1-^{13}C]phenylalanine oxidation did not differ between the 5 test diets and that essential amino acids were supplied in excess of the estimate dietary requirements for preterm infant formula (19). Although the dietary intake of formula 1 (170 mL/(kg·d)) was higher than formula 4 (163 mL/(kg·d), it did not significantly differ. We do not believe that this had a major influence on the obtained results. The higher intake of formula 1 could have resulted in a lower phenylalanine oxidation rate because of a slightly higher cyst(e)ine intake compared with formula 2. However, we did not find a difference in the phenylalanine oxidation.

The current guidelines on requirements of individual amino acids for infants are based on the factorial method. To calculate the deposit of each amino acid, growth rate is assumed to be constant at 15 g/(kg·d) for a fetus from 900 to 2400 g and protein retention at 70% of total protein intake. Furthermore, the protein maintenance requirement in preterm infants ranges from 0.55 to 0.75 g/(kg·d) (20). Fetal amino acid accretion during normal intrauterine growth

was determined by Widdowson (21). Cyst(e)ine accretion by the human fetus was not determined, however, and thus the requirement could not be estimated by the factorial approach.

Little is known, too, about the biosynthetic capacity of non-essential amino acids in preterm infants. In the early 1970s several investigators reported that the transsulfuration pathway might be immature in preterm infants because of limited enzyme activity of cystathionase (1-3). Some found cystathionase activity to be absent in fetal liver tissues (2, 3). Zlotkin and Andersson (1) showed that cystathionine activity was limited but not isolated to the liver and was also present in extrahepatic tissues (kidneys and adrenals). They also found in term and preterm infants that this activity increased after birth. Furthermore, Stegink and Den Besten (22) showed that plasma cysteine concentrations in adult humans fed a cystine deficient diet intragastrically were significantly higher than in those fed the same diet intravenously. This finding suggests an important role in cyst(e)ine production for the gut and was confirmed in animal experiments. Neonatal piglets fed a cysteine-free diet enterally showed significantly higher plasma cysteine concentrations than did parenterally fed piglets (23). In addition, dietary methionine and plasma cysteine concentrations were positively correlated in enterally fed piglets but not in parenterally fed piglets.

Earlier studies that investigated whether cyst(e)ine is an essential amino acid in preterm infants were all performed during parenteral nutrition. Several of those studies reported low plasma cysteine concentrations, with or without cysteine supplementation (24-26). Pohlandt (26) observed no differences in plasma cystine concentrations between preterm infants receiving only glucose intravenously and preterm infants fed a mixture of synthetic amino acids free from cystine. In both groups cystine plasma concentrations were low, < 5 μmol/L, indicating limited endogenous synthesis from methionine. Furthermore, Viňa et al. (25) and Miller et al. (27) reported impaired cysteine metabolism in premature infants on total parenteral nutrition. All those studies indicate that limited cystathionase activity makes cyst(e)ine a conditionally essential amino acid in preterm infants. Zlotkin et al. (28), nevertheless, reported that cysteine supplementation did not affect growth rate and nitrogen balance in parenterally fed term and preterm infants. It did slightly increase urinary 3-methylhistidine excretion, however, suggesting that either muscle protein catabolism or muscle mass had increased. Also Malloy et al. (29) did not find an improved nitrogen balance with cysteine supplementation, although plasma-free cyst(e)ine concentration increased. Contradictorily, findings from a recent study suggest that the transsulfuration pathway produces sufficient amounts of cysteine in the parenterally fed preterm infant (30).

Our study was performed when neonates received full enteral feeding supplied with a generous amount of methionine and shows that the cyst(e)ine synthesis pathway is active and sufficient at this time. We studied the indicator oxidation during 5 h when infants were continuously fed. The standard feeding regimen of these infants in our neonatal intensive care unit dictates feeding every hour or every 2 h, depending on clinical considerations. Thus, we studied neonates under physiologic circumstances. In our view, therefore, the absence of a postabsorptive state in these preterm infants does not necessitate the study of oxidation during 24 h.

In conclusion, our findings reject our hypothesis that cyst(e)ine is a conditionally essential amino acid in 1-mo-old fully enterally fed LBW preterm infants with a mean GA of 33 wk. These findings show indirectly that activity of the enzyme cystathionase is sufficient to synthesize adequate amounts of cyst(e)ine in these infants supplied with a generous amount of dietary methionine.

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The author's responsibilities were as follows - MAR collected and analyzed the data and wrote the manuscript; RHTvB, HMAdB and ACMD recruited all patients; GV analyzed the data; JBvG designed the study and provided helpful comments on the manuscript. None of the authors had any financial or personal interest in any company or organization sponsoring the research.

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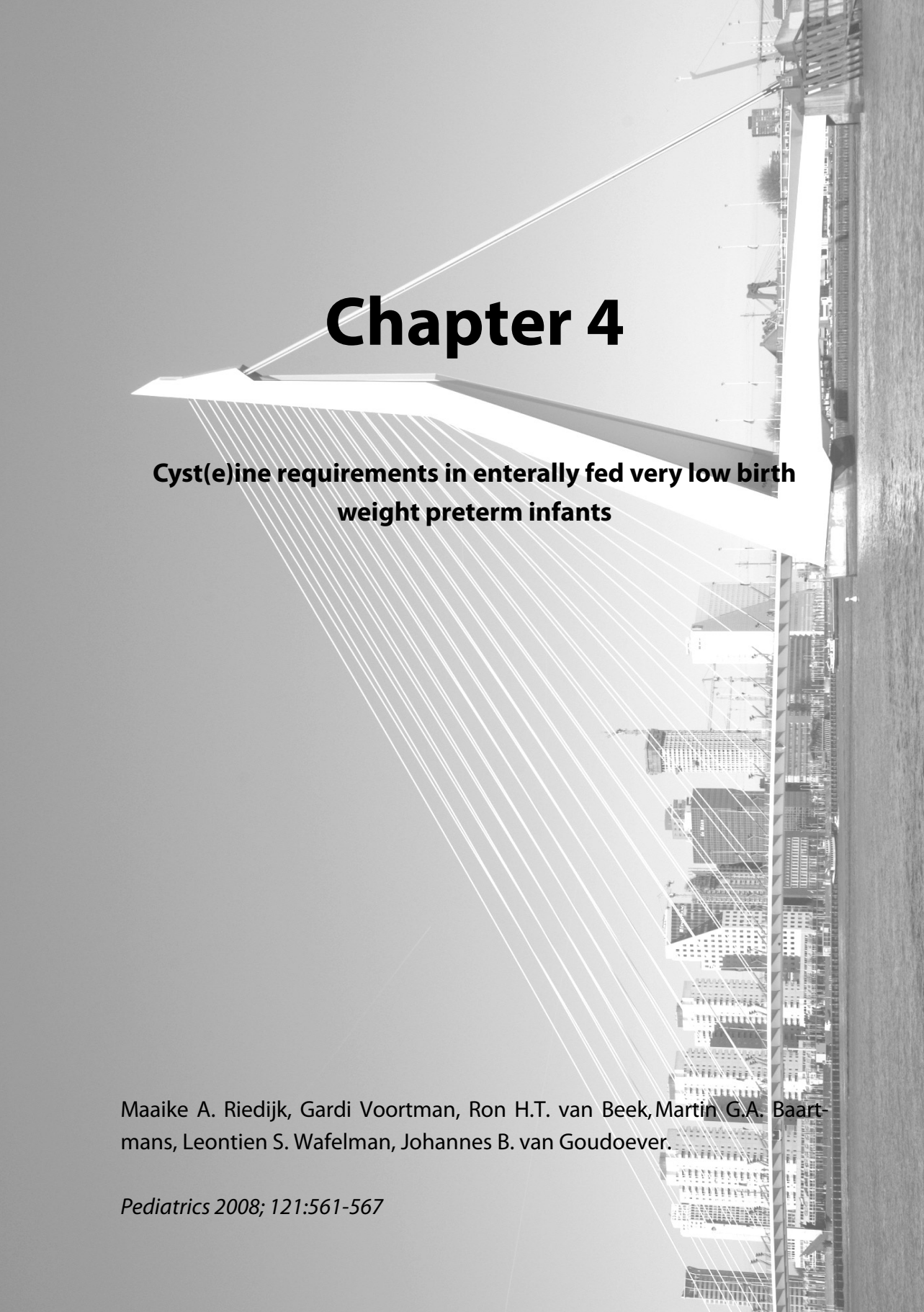

Chapter 4

Cyst(e)ine requirements in enterally fed very low birth weight preterm infants

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ABSTRACT

Optimal nutrition is of utmost importance for the preterm infant's later health and developmental outcome. Amino acid requirements for preterm infants differ from those for term and older infants, because growth rates differ. Some nonessential amino acids, however, cannot be sufficiently synthesized endogenously. Cyst(e)ine is supposed to be such a conditionally essential amino acid in preterm infants.

The objective of this study was to determine, at 32 and 35 weeks' postmenstrual age, cyst(e)ine requirements in fully enterally fed very-low-birth-weight preterm infants with gestational ages of < 29 wk.

Infants were randomly assigned to 1 of the 5 graded cystine test diets that contained generous amounts of methionine. Cyst(e)ine requirement was determined with the indicator amino acid oxidation technique ([1-^{13}C]phenylalanine) after 24-h adaptation.

Fractional [1-^{13}C]phenylalanine oxidation was established in 47 very-low-birth-weight preterm infants (mean gestational age: 28 ± 1wk SD; birth weight: 1.07 ± 0.21 kg SD). Increase in dietary cyst(e)ine intake did not result in a decrease in fractional [1-^{13}C]-phenylalanine oxidation.

These data do not support the hypothesis that endogenous cyst(e)ine synthesis is limited in very-low-birth-weight preterm infants with gestational ages of < 29 wk, both at 32 and 35 wk postmenstrual age. It is safe to conclude that cyst(e)ine requirement is < 18 mg/(kg·d) in enterally fed very-low-birth-weight preterm infants who are older than 32 wk postmenstrual age and whose methionine intake is adequate. Therefore, cyst(e)ine is probably not a conditionally essential amino acid in these infants.

INTRODUCTION

Note: Cyst(e)ine is used throughout to designate any undefined combination of cysteine and cystine.

Early nutrition is pivotal for preterm infants' survival but also has profound influence on their later developmental and intelligence outcomes (1, 2). The quantity of administered essential amino acids is also of relevance; however, some nonessential amino acids are considered conditionally essential during specific circumstances (eg, rapid growth or critical illness) (3). Endogenous synthesis then will temporarily not be sufficient to meet the requirement. Cyst(e)ine is believed to be such a conditionally essential amino acid in preterm infants, because preterm infants show biochemical immaturity of cystathionase (EC 4.4.1.1), the enzyme catalyzing the final step in cyst(e)ine synthesis pathway (ie, transsulfuration pathway) (4-6). Cyst(e)ine is a sulfur-containing amino acid that is nonessential in humans. It is synthesized de novo from methionine, which is the only essential sulfur-containing amino acid, and from serine. Cyst(e)ine has several important metabolic functions. First, like all other amino acids, it is involved in growth and protein synthesis. Furthermore, it is one of the amino acid components of the tripeptide glutathione, an important intracellular antioxidant. It is also a precursor for taurine and sulfate.

It is, therefore, important to know the exact cyst(e)ine requirements of preterm infants at various postnatal ages, considering that cystathionase maturation occurs postnatally. Previous estimates of amino acid requirements were based on less accurate methods than are currently available. We performed a study aiming at estimating cyst(e)ine requirements in very-low-birth-weight (VLBW) preterm infants at 4 and 8 weeks postnatally, using the indicator amino acid oxidation (IAAO) method. This method was used to reestimate individual essential amino acid requirements in adults (7-9) and was introduced by Zello et al. (10). We hypothesized that cyst(e)ine is an essential amino acid for VLBW preterm infants early in life; however, it depends on postmenstrual age (PMA), and it becomes a nonessential amino acid after maturation of the transsulfuration pathway.

METHODS

Subjects

Patient who were eligible for the study were VLBW infants who were born at gestational age (GA) of < 29 wk and admitted to the neonatal intensive care unit of the Erasmus MC-Sophia Children's Hospital (Rotterdam, the Netherlands) in the first days of life. At the time of the study, a number of patients had already been transferred to affiliated hospitals in the region: Amphia Hospital (Breda), Albert Schweitzer Hospital (Dordrecht) or the Medical Center Rijmond-Zuid (Rotterdam), all in the Netherlands. In that case, the study was conducted in the hospital of relevance.

We determined cyst(e)ine requirements in patients who were aged 1 month (group 1) and patients who were aged 2 months (group 2) postnatally. They needed to be clinically stable

and were excluded in case of congenital or gastrointestinal diseases. Infants were enrolled in the study when they tolerated full enteral feeding (> 150 mL/(kg·d)). Feeding was either completely through a nasogastric feeding tube or partly by bottle, depending on postconceptual age. During the study, all infants were breathing spontaneously for at least 8 h. The study protocol was approved by the Central Committee on Research Involving Human Subjects, the Erasmus MC institutional review board and review boards from the affiliated hospitals. Written informed consent was obtained from both parents of each patient.

Study formula

In this study, we used 5 elemental study formulas that contained graded cyst(e)ine concentrations: 11, 22, 32, 43 and 65 mg cyst(e)ine/100 mL (Xcys/Neocate; Nutricia Nederland BV, Zoetermeer, the Netherlands/SHS International, Liverpool, United Kingdom). The cyst(e)ine content was monitored by SHS International. The study formulas were not totally isonitrogenous. Except for cyst(e)ine concentration, the 5 formulas did not differ as to amino acid composition. Methionine intake was similar for all formula groups (71 mg/(kg·d)) and was supplied generously according to the estimated methionine requirement for preterm infants (48-69 mg/(kg·d)) (11).

Study design and tracer protocol

Infants who were eligible for the study were included and cyst(e)ine requirement was determined ~ 1 month after birth (PMA range: 30-32 wk) or 2 months after birth (PMA range: 35-37 wk). Infants were randomly assigned to at least one of the study formulas. The study diet was initiated 24 h before start of the study so that the patient could adapt to the diet. Cyst(e)ine intake and the dietary intake was not changed until the tracer protocol was finished. The adaptation period of 24 h was selected according to studies by the group of Pencharz (12, 13).

All patients received ~ 170 mL/(kg·d) formula to ensure that all other essential amino acids, particularly methionine, were in excess and, therefore, not limiting for protein synthesis. Before the introduction of the study formula, almost all infants received their mother's (expressed) breast milk, and only a few infants received standard preterm formula (Nenatal; Nutricia, Zoetermeer, the Netherlands). The cyst(e)ine concentration of breast milk varies widely, but the preterm formula provided 35 mg cyst(e)ine/100 mL.

The IAAO method is based on a labeled essential amino acid that is different from the test amino acid. This indicator is independent of the different intake levels of the test amino acid. If the test amino acid is deficient in the diet, then this will limit overall protein synthesis and all other essential amino acids will be oxidized. As dietary intake of the test amino acid increases, oxidation of the indicator will decrease linearly until requirement of the test amino acid is met. We chose [1-^{13}C]phenylalanine as the indicator (14).

After 24-h adaptation, patients received a primed (10 µmol/(kg)) continuous (10 µmol/(kg·h)) enteral infusion of [^{13}C]bicarbonate (sterile pyrogen free, 99% atom percent excess (APE); Cambridge Isotopes, Woburn, MA) for 2.5 h to quantify individual CO_2 production. We

infused the tracer enterally to minimize invasiveness of the experiment. This method has been validated by our group (15). The labeled sodium bicarbonate infusion was directly followed by a primed (30 µmol/(kg)) continuous (30 µmol/(kg·h)) enteral infusion of [1-^{13}C]-phenylalanine (93% APE; Cambridge Isotopes) for 5 h. One hour before start of the oxidation study, the feeding regimen was changed into continuous dripfeeding. Enterally infused tracer was mixed with the study formula and infused continuously by an infusion pump through the nasogastric tube.

Breath samples were obtained using the direct sampling method described by Van der Schoor et al (16). In brief, a 6F gastric tube (6 Ch Argyle; Cherwood Medical, Tullamore, Ireland) was inserted 1 to 1.5 cm into the nasopharynx and end-tidal breath was taken slowly with a syringe connected at the end. Collected air was transferred into 10-mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and stored at room temperature until analysis. Baseline samples were obtained 15 and 5 min before start of tracer infusion. Duplicate ^{13}C-enriched breath samples were first collected every 30 min, and every 15 min during the last 45 min of tracer infusion.

Analytical methods and calculations

$^{13}CO_2$ isotopic enrichment in expired air was measured by isotope ratio mass spectrometry (ABCA; Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands) and expressed as APE above baseline (16). APE was plotted relative to [1-^{13}C]phenylalanine infusion time.

Estimated body CO_2 production (mmol/(kg×h) was calculated as described previously (15). The rate of fractional [1-^{13}C]phenylalanine oxidation was calculated as follows:

$$\text{Fractional phenylalanine oxidation (\%)} = [IE_{PHE} \times i_B]/[i_{PHE} \times IE_B] \times 100$$

where IE_{PHE} is the ^{13}C isotopic enrichment in expired air during [1-^{13}C]phenylalanine infusion (APE), i_B is the infusion rate of [^{13}C]bicarbonate (µmol/(kg·h)), i_{PHE} is the infusion rate of [1-^{13}C]-phenylalanine (µmol/(kg·h)) and IE_B is the ^{13}C isotopic enrichment in expired air during [^{13}C]-bicarbonate infusion.

Statistical analysis

Descriptive data are expressed as mean ± SD. The steady state of $^{13}CO_2$ release in expired breath during the [^{13}C]bicarbonate and [1-^{13}C]phenylalanine infusions were achieved when the linear factor of the slope was found not to be significantly different from zero ($p > 0.05$). The cyst(e)ine requirement was determined with the use of the IAAO method. The indicator oxidation rate was plotted against varying dietary cyst(e)ine intakes (mg/(kg·d)). The inflection or breakpoint in the indicator oxidation rate represents the physiological cyst(e)ine requirement (17).

Data were analyzed with the use of mixed model analysis of variance in SPSS software (version 14.0; SPSS Inc. Chicago, IL), while encoding the patients who participated twice with the same number. Repeated measures analysis of variance was performed on primary and derived variables to assess the effects of dietary intake and of patients. Regression analysis

was performed to analyze oxidation rates. Power calculation revealed that assuming 5 formula groups with a group variance of 16, an intergroup variance of 5.5, and a power of 80%, a breakpoint should be detected with 5 patients per group. Statistical significance was assumed at 5% level of significance ($p \leq 0.05$).

RESULTS

We determined the cyst(e)ine requirements in VLBW infants ~ 1 month after birth (group 1) and 2 months after birth (group 2). We included in total 47 VLBW infants: 20 infants in group 1 and 27 infants in group 2. Patient characteristics are depicted in Tables 1 and 2. Seven infants were studied at both time points, and in each group three children participated twice and were assigned to 2 different formulas. Infants were randomly assigned to one or two study formulas providing ~ 18, 36, 54, 72 or 109 mg cystine/(kg·d) at an intake of 168 mL/(kg·d). The mean GA of all infants in both groups was 28 ± 1 wk, and mean birth weight was 1.07 ± 0.21 kg (data not shown). Weight gain rate of the infants during the days before the study was > 10 g/(kg·d).

We performed 23 measurements in 20 infants (14 male, 6 female) at mean PMA 32 ± 0 wk (range: 31-32 wk). Numbers of patients who received each formula are given in Table 1, either 4 or 5 per formula. We performed 6 measurements per formula in 27 infants (18 male, 9 female) at mean PMA 35 ± 1 wk (range: 35-38 wk), so in total we studied 30 [1-^{13}C]-phenylalanine oxidation rates in this group (Table 2).

In both groups, GA, birth weight and study weight did not differ among the 5 formula groups (Tables 1 and 2); however, by chance, in group 1, the PMA of formula 2 was slightly higher compared with formula 5 ($p = 0.04$). We do not believe that this would have had any affect on the results because of the small difference in PMA and because the patients' cyst(e)ine intake was randomly selected. The total enteral intake of both groups did not differ among the 5 formula groups (group 1: $p = 0.14$, group 2: $p = 0.08$).

To compare outcome parameters between the five formulas within each group, we corrected the model for gender, study age, and study weight. In both groups, the baseline ^{13}C enrichment in expired breath did not differ among the formulas (Table 3). Each patient reached plateau during both [^{13}C]bicarbonate and [1-^{13}C]phenylalanine tracer infusions. For 6 patients (group 2) who received 72 mg/(kg·d) cystine, the ^{13}C enrichments in expired breath during the infusion of [1-^{13}C]phenylalanine are shown in Figure 1.

In group 1, the fractional oxidation rates of [1-^{13}C]phenylalanine among the 5 formulas did not significantly differ ($p = 0.19$). Regression did not show a linear decease of the fractional oxidation rate when cyst(e)ine intakes increased (Figure 2). The regression line was not different from zero ($p = 0.09$) but showed a slightly positive trend in oxidation rate with increasing cyst(e)ine intake ($p = 0.05$). This trend seems to refute our hypothesis that the oxidation rate would decrease with increasing cyst(e)ine intake. In group 2, the fractional [1-^{13}C]phenylalanine oxidation did not differ among the 5 different cyst(e)ine intake groups ($p = 0.84$). Again, regression of the data did not show a linear decrease in oxidation of the

Table 1. Patient characteristics at ~ 1 months' PMA for group 1.

Diet	No. of trials	GA	PMA	Birth weight	Study weight	Enteral intake	Cyst(e)ine intake
		wk	wk	kg	kg	mL/(kg·d)	mg/(kg·d)
1	5	27 ± 1	31 ± 1	1.14 ± 0.28	1.31 ±0.18	167 ± 2	18 ± 0
2	5	28 ± 2	32 ± 0	1.20 ±0.13	1.53 ± 0.17	168 ± 0	36 ± 0
3	4	27 ± 1	32 ± 0	1.06 ± 0.05	1.45 ± 0.22	166 ± 2	54 ± 1
4	5	27 ± 1	32 ± 0	1.03 ± 0.10	1.38 ± 0.34	168 ± 0	72 ± 0
5	4	28 ± 1	31 ± 1	1.13 ± 0.17	1.33 ± 0.12	167 ± 2	108 ± 2
Mean		27	32	1.12	1.40	167	
SD		1	0	0.16	0.22	1	

Data are mean ± SD (n = 20, measurements = 23). GA, $p = 0.99$; PMA, $p = 0.04$; Birth weight, $p = 0.61$; Study weight, $p = 0.66$.

Table 2. Patient characteristics ~ 2 months' PMA for group 2.

Diet	No. of trials	GA	PMA	Birth weight	Study weight	Enteral intake	Cyst(e)ine intake
		wk	wk	kg	kg	ml/(kg·d)	mg/(kg·d)
1	6	28 ± 1	35 ± 1	1.03 ± 0.26	2.21 ± 0.43	168 ± 2	18 ± 0
2	6	29 ± 1	36 ± 1	1.14 ±0.18	2.09 ± 0.47	168 ± 1	36 ± 0
3	6	29 ± 0	35 ± 0	1.12 ± 0.22	1.78 ± 0.22	166 ± 3	53 ± 1
4	6	28 ± 2	36 ± 1	0.97 ± 0.25	2.13 ± 0.35	168 ± 1	72 ± 0
5	6	27 ± 2	35 ± 1	0.99 ± 0.26	2.09 ± 0.54	167 ± 1	108 ± 1
Mean		28	35	1.05	2.06	167	
SD		1	1	0.23	0.41	2	

Data are mean ± SD (n = 27, measurements = 30). GA, $p = 0.33$; PMA, $p = 0.30$; Birth weight, $p = 0.53$; Study weight, $p = 0.35$.

Table 3. Whole-body CO_2 production rates and fractional oxidation rates during [1-^{13}C]phenylalanine infusion at 5 different cyst(e)ine intakes .

Group	Cyst(e)ine intake	Baseline $^{13}CO_2$	CO_2 production	$^{13}CO_2$ [^{13}C]bicarb	$^{13}CO_2$ [1-^{13}C]phe	Fractional oxidation [1-^{13}C]phe
	mg/(kg·d)	PDB[1]	mmol/(kg·d)[2]	APE[3]	APE	%[4]
1	18 (n=5)	-16.49 ± 0.54	30.42 ± 2.11	0.0329 ± 0.0025	0.0182 ± 0.0024	18.46 ± 1.79
1	36 (n=5)	-17.32 ± 0.73	34.17 ± 5.31	0.0305 ± 0.0045	0.0192 ± 0.0033	21.64 ± 3.34
1	54 (n=4)	-16.97 ± 0.55	31.93 ± 1.46	0.0301 ± 0.0014	0.0171 ± 0.0055	18.29 ± 5.55
1	72 (n=5)	-17.29 ± 0.29	31.24 ± 1.70	0.0315 ± 0.0017	0.0187 ± 0.0042	19.76 ± 4.23
1	109 (n=5)	-16.86 ± 0.51	32.89 ± 3.76	0.0307 ± 0.0038	0.0226 ± 0.0057	24.34 ± 3.77
2	18 (n=6)	-17.58 ± 0.43	30.15 ± 2.89	0.0333 ± 0.0033	0.0182 ± 0.0042	18.11 ± 3.52
2	36 (n=6)	-17.32 ± 0.51	34.53 ± 7.04	0.0296 ± 0.0049	0.0181 ± 0.0049	20.16 ± 3.51
2	54 (n=6)	-17.60 ± 1.53	31.62 ± 2.94	0.0316 ± 0.0031	0.0185 ± 0.0054	19.50 ± 5.07
2	72 (n=6)	-17.62 ± 1.03	31.41 ± 2.54	0.0315 ± 0.0025	0.0175 ± 0.0043	18.52 ± 4.33
2	109 (n=6)	-17.79 ± 0.79	31.59 ± 2.93	0.0314 ± 0.0032	0.0190 ± 0.0046	20.35 ± 5.24

Data are mean ± SD. Baseline $^{13}CO_2$ is expressed as Pee Dee Belemnite (PDB). $^{13}CO_2$ indicates enrichment of ^{13}C in expired air in APE. [^{13}C]bicarb, labeled sodium bicarbonate; phe, phenylalanine. Group 1, VLBW infants who were born at a mean GA of 27 wk and studied at a mean PMA of 32 wk; group 2, VLBW infants who were born at a mean GA of 28 wk and studied at a mean PMA of 35 wk. [1] Group 1, $p = 0.19$, group 2, $p = 0.98$, [2] Group 1, $p = 0.27$, group 2, $p = 0.57$, [3] Group 1, $p = 0.57$, group 2, $p = 0.57$, [4]Group 1, $p = 0.19$, group 2, p, $= 0.84$.

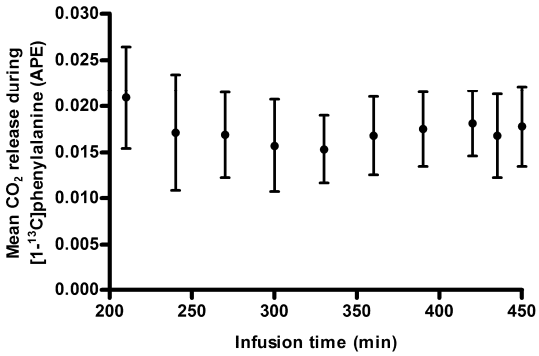

Figure 1. Mean ^{13}C enrichments in expired breath during enteral [1-^{13}C]phenylalanine infusion in 6 infants (mean PMA: 35 wk) who received 72 mg/(kg·d) cyst(e)ine (formula 4).

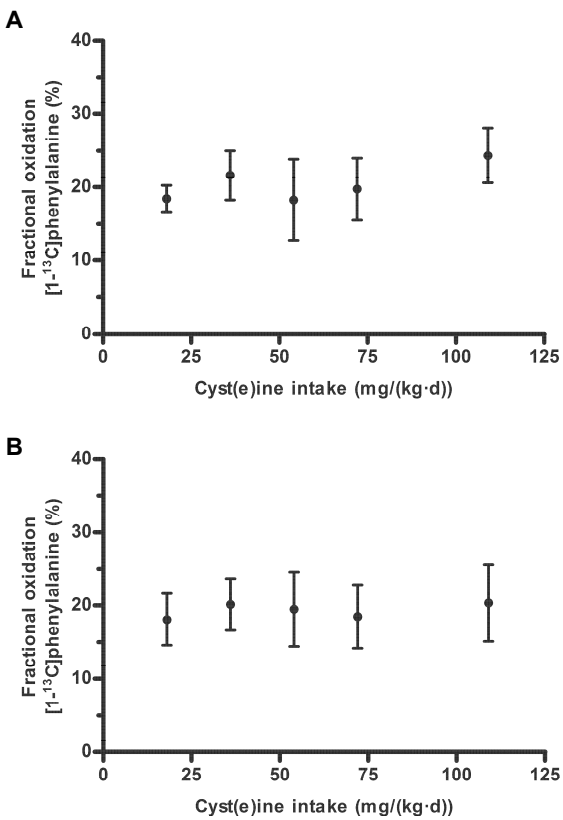

Figure 2. Relationship between the cystine intake and the individual fractional [1-^{13}C]phenylalanine oxidation in VLBW infants with a mean GA of 28 wk at 32 (A) and 35 (B) weeks' PMA. A, The fractional oxidation of [1-^{13}C]-phenylalanine in VLBW infants at ~ 32 weeks' PMA (group 1). Five, 5, 4, 5, and 4 measurements were taken for cyst(e)ine intakes of 18, 36, 54, 72 and 109 mg/(kg·d), respectively. B, The fractional oxidation of [1-^{13}C]-phenylalanine in VLBW infants at ~ 35 weeks' PMA (group 2). Six measurements were taken for each cyst(e)ine intake.

indicator (Figure 2), and the regression line was also not significantly different from zero. A trend in the formula could not be detected (p = 0.24). Consequently, in both groups, an inflection of the curve is missing and no breakpoint could be calculated.
These results suggest that the cyst(e)ine requirement in VLBW infants who are older than 32 PMA is already met under these circumstances (ie, at 18 mg cyst(e)ine/(kg·d) intake together with 71 mg/(kg·d) methionine at a total enteral intake of 170 mL/(kg·d)). At the intakes of 18 to 109 mg/(kg·d), cyst(e)ine is not the limiting amino acid for protein synthesis and is therefore not deficient in the diet.

DISCUSSION

In this study, application of the IAAO method showed that a cyst(e)ine intake of 18 mg/(kg·d) did not limit endogenous cyst(e)ine synthesis and that the cyst(e)ine requirement was < 18 mg/(kg·d). Hence, it is presumably safe to say that cyst(e)ine is not a conditionally essential amino acid in fully enterally fed VLBW preterm infants who are older than 32 weeks' PMA.
The IAAO method is based on the assumption that essential amino acids participate between protein synthesis and oxidation (18). If one essential amino acid is deficient in the diet, then this will limit overall protein synthesis and all remaining essential amino acids are in excess and thus will be oxidized. Generous amounts of each essential amino acid must be applied, therefore, except for the one under study. We could not determine a decrease in fractional [1-^{13}C]phenylalanine oxidation with increasing cystine intake. Thus, an amino acid other than cystine or other co-factors for protein synthesis could have been limiting and hence no change in oxidation rate could be detected; however, weight gain rates in both groups were not compromised, indicating no major limitations for protein synthesis. In addition, the amount of methionine provided by a daily intake of 168 mL/(kg·d) was 70 mg/(kg·d), ~ 20% in excess of the methionine requirement estimated on fetal accretion rate and obligatory losses (59 mg/(kg·d)) (19). We propose, therefore, that methionine intake is not the limiting factor for endogenous cyst(e)ine synthesis. Another explanation for our finding might lie in the duration of the adaptive period to a different cystine intake. At 24 h it might have been too short; however, Zello and colleagues (18, 20) did not see an effect of previous adaptation to various levels of the test amino acid and suggested 4 h to be enough to establish a new steady state.
Over the years, various methods have been used to estimate individual amino acid requirements (eg, the nitrogen balance method, growth rate, plasma amino acid patterns, the factorial approach). In 1971 Snyderman et al. (21) reported amino acid requirements for neonates on the basis of nitrogen balance and weight gain rate, yet nitrogen balance usually overestimates nitrogen retention, and growth rate also depends on factors other than amino acid intake. Moreover, 7 to 10 days' adaptation is needed to establish nitrogen equilibrium (22). Because present clinical practice does not accept maintaining neonates on either deficient or excess amino acid intakes for a minimum of 7 d, no such requirement studies

have been reported in preterm infants since then.

Current dietary requirement estimates for humans so far are based on the factorial approach (11). For preterm infants, this approach uses data on body composition of the fetus in utero of approximately the same age. These data are derived from body carcass analysis of stillborn preterm infants, some born > 100 years ago (23-25). In many cases, GA of the analyzed fetuses was not accurately known; therefore, not all data could serve as standard reference (26). Because fetal accretion rate of cyst(e)ine is not available, the current cyst(e)ine requirement for preterm infants (66-95 mg/(kg·d) is based on the minimum and maximum amounts of each amino acid present in the amounts of breast milk protein corresponding to the recommended minimum and maximum protein contents (g/503 kJ) of 3.0 and 4.3 g, respectively (27). This estimation, however, does not take into account the influence of postnatal maturation.

Until now, cyst(e)ine was believed to be a conditionally essential amino acid in preterm infants. Snyderman (21) was the first to show that cyst(e)ine might be required for preterm infants. She found lower rates of nitrogen retention and weight gain in 2- to 4-month-old infants who were born preterm and enterally fed a synthetic diet without cyst(e)ine. The cyst(e)ine intakes of 44 or 66 mg/(kg·d) did not restore nitrogen retention and weight gain to control values. She recommended a minimal intake of 85 mg cyst(e)ine/(kg·d); however, because she did not provide methionine intake, this recommendation might be overestimated in view of the risk of inadequate methionine intake. Several in vitro studies then reported that the enzyme cystathionase was absent in fetal liver in these infants, in contrast to term infants (4-6). Cystathionase activity seems to be a postnatal phenomenon, reaching mature levels at ~ 3 months of age (5, 6). Cyst(e)ine requirement in these infants thus would depend on GA and should decrease with postnatal age. Obviously, we did not confirm this hypothesis.

Several in vivo studies demonstrated low plasma cyst(e)ine concentrations in preterm infants with or without cyst(e)ine supplementation, suggesting that limited cystathionase activity had impaired cyst(e)ine synthesis (28-31). Conversely, Zlotkin et al. (32) did not find differences in nitrogen balance for the parenterally fed term and preterm infants with or without cysteine supplementation. They showed slightly higher urinary 3-methylhistidine excretion in cysteine supplemented infants but failed to detect an evident relation. Although Malloy et al. (33) showed that cysteine supplementation increased free cysteine plasma concentration and sulfur balance in preterm infants, it did not improve nitrogen retention. A study in parenterally fed VLBW infants who received an isotopically labeled glucose infusion showed incorporation of isotopic label in plasma cysteine and in hepatically derived apo B-100 cysteine (34). In agreement with our results, the authors concluded that these infants were certainly capable of sufficient endogenous cyst(e)ine synthesis, which was directly related to birth weight .

Cyst(e)ine is an important sulfur-containing amino acid. Indispensable for protein synthesis, it also serves as a significant precursor for glutathione synthesis. If cyst(e)ine is a conditionally essential amino acid in preterm infants, then it could be the limiting factor for adequate glutathione production. Preterm birth and critical illness including oxygen supplementation

might lead to higher glutathione requirement might be increased in these infants. If cyst(e)ine concentration is inadequate, then glutathione quantity might be insufficient to prevent oxidative stress; however, Shew et al. (34) consider the minimum capacity for cysteine synthesis enough to counteract oxidative stress. For confirmation of this statement, future studies should investigate incorporation of quantities of cysteine in glutathione in both parenterally and enterally fed preterm infants.

A limitation of this study is that we did not include a formula providing for cyst(e)ine intake of < 18 mg/(kg·d). The formulas used were based on current nutrient recommendations for preterm formula (27). At the onset of the study, we decided not to incorporate a study formula without cyst(e)ine for ethical reasons. Whether cyst(e)ine requirement is > 0 but < 18 mg/(kg·d) has to be determined with the aid of a cyst(e)ine-free formula yet with sufficient methionine.

From the findings of this study, we may safely conclude that the cyst(e)ine requirement for enterally fed VLBW preterm infants who are older than 32 weeks' PMA is < 18 mg/(kg·d), provided that methionine intake is adequate.

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Sulfur amino acid metabolism

Chapter 5

Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract

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ABSTRACT

Methionine is an indispensable sulfur amino acid that functions as a key precursor for the synthesis of homocysteine and cysteine. Studies in adult humans suggest that splanchnic tissues convert dietary methionine to homocysteine and cysteine by means of transmethylation and transsulfuration, respectively. Studies in piglets show that significant metabolism of dietary indispensable amino acids occurs in the gastrointestinal tissues (GIT), yet the metabolic fate of methionine in GIT is unknown. We show here that 20% of the dietary methionine intake is metabolized by the GIT in piglets implanted with portal and arterial catheters and fed milk formula. Based on analyses from intraduodenal and intravenous infusions of [1-^{13}C]methionine and [2H3]methionine, we found that the whole-body methionine transmethylation and remethylation rates were significantly higher during duodenal than intravenous tracer infusion. First-pass splanchnic metabolism accounted for 18% and 43% of the whole-body transmethylation and remethylation, respectively. Significant transmethylation and transsulfuration was demonstrated in the GIT, representing ~27% and ~23% of whole-body fluxes, respectively. The methionine used by the GIT was metabolized into homocysteine (31%), CO2 (40%), or tissue protein (29%). Cystathionine ß-synthase mRNA and activity was present in multiple GITs, including intestinal epithelial cells, but was significantly lower than liver. We conclude that the GIT consumes 20% of the dietary methionine and is a significant site of net homocysteine production. Moreover, the GITs represent a significant site of whole-body transmethylation and transsulfuration, and these two pathways account for a majority of methionine used by the GITs.

INTRODUCTION

The sulfur amino acids, methionine, homocysteine, and cysteine, have attracted considerable interest in the last decade. Methionine is an indispensable amino acid and is transmethylated intracellularly to homocysteine via S-adenosylmethionine (SAM), an important methyl donor for most biological methylation reactions (1). Homocysteine can be further catabolized to cysteine through transsulfuration, which is regulated by the enzymes cystathionine β-synthase (CBS) (EC 4.2.1.22) and cystathionine γ-lyase (EC 4.4.1.1). Homocysteine can also be used for methionine synthesis through remethylation. Consequently, homocysteine represents a critical regulatory control point for methionine and cysteine synthesis and folate metabolism.

Homocysteine is normally present in human plasma at low concentrations. However, elevated plasma homocysteine concentrations have important implications for human health and disease. Clinical studies indicate that hyperhomocysteinemia is strongly associated with increased risk of cardiovascular disease, with ischemic and hemorrhagic stroke in infants and children (2-4), and, more recently, Alzheimer's disease and other neurological diseases in adults (5). Genetic defects in methionine metabolism related to methionine synthase, $N^{5,10}$-methylene-tetrahyrdofolate, also result in neural dysfunction, mental retardation, and pregnancy complications (6-8). Recent studies have linked homocysteine, folate, and DNA methylation with gastrointestinal diseases, namely inflammatory bowel disease and colon cancer (9-13). The underlying mechanism linking homocysteine to inflammatory disease may be the induction of leukocyte adhesion molecules and proinflammatory cytokines in vascular endothelial cells (10).

Methionine is also a precursor for cysteine, which plays a key role in cellular protein function and redox status by virtue of its thiol (-SH) moiety. In addition, it also serves as a precursor of glutathione, a major cellular antioxidant, as well as CoA, taurine, and inorganic sulfur. Several tissues in the body are capable of cysteine synthesis (14), which is why cysteine is considered nutritionally dispensable. However, increasing evidence that oxidant stress is linked to many disease pathologies and aging has focused attention on the antioxidant functions of cysteine and its product, glutathione, leading some to suggest that cysteine is a conditionally indispensable dietary nutrient (15-17).

Most cells in the body are capable of transmethylation and remethylation, yet homocysteine metabolism via transsulfuration seems limited to certain tissues. Early studies by Mudd et al. (18) showed that CBS and cystathionine γ-lyase are active mainly in human liver tissue, and to a lesser extent, in extra hepatic tissues including pancreas, kidney, small intestine, brain, and lung. Stegink and Den Besten (19) first reported in vivo evidence to implicate splanchnic tissues as a key site of methionine transsulfuration by demonstrating that plasma cysteine concentrations are significantly higher in human subjects fed a methionine-containing, cystine-free diet enterally versus intravenously. However, subsequent metabolic studies with stable isotopic tracers also have indirectly suggested that a substantial fraction of the dietary methionine undergoes transmethylation and remethylation during first-pass splanchnic metabolism in adult humans (20, 21). More recent studies show that the whole-body

methionine requirement and circulating homocysteine concentrations are significantly higher in piglets fed enterally than parenterally, demonstrating that splanchnic methionine metabolism is nutritionally significant (22, 23).

Despite the clear evidence that splanchnic tissues play a major role in methionine transmethylation and transsulfuration, the relative contribution of the gastrointestinal tissues (GIT) remains unclear. Our previous studies have shown that GIT extensively metabolize dietary indispensable amino acids, namely lysine, threonine, and leucine (24-26). In the case of methionine, our studies and those of others suggest that substantial metabolism and even oxidation of dietary methionine occurs in the gut (26, 27). However, the extent of transmethylation and transsulfuration in the GIT and its relative contribution to whole-body methionine metabolism has not been established. In this report, we have coupled our piglet model of arteriovenous balance across the GIT with an established stable isotopic tracer approach using [1-^{13}C and methyl-$^{2}H_3$]methionine to investigate the metabolic fate of methionine in the gut and the contribution to the whole-body rates of transmethylation and transsulfuration. We hypothesized that there is substantial transmethylation, transsulfuration, and remethylation of dietary methionine by GIT and that dietary rather than systemically derived methionine is preferentially metabolized.

RESULTS

Whole-body methionine metabolism

Mean weight of the animals on the first and second days of the experiment were 6.74 ± 1.89 kg and 7.52 ± 1.99 kg, respectively. No significant differences between intravenous (IV) and intraduodenal (ID) infusions were observed in mean whole-body CO_2 production (IV 46.55 ± 1.16 mmol/(kg·h) and ID 45.50 ± 0.97 mmol/(kg·h)) and CO_2 production by the portal drained viscera was 6.95 ± 0.57 mmol/(kg·h) IV and 6.73 ± 0.55 mmol/(kg·h) ID. As shown in Figure 1, steady-state of plasma isotopic enrichment (expressed as mole percent excess, MPE) of [1-^{13}C]methionine and [$^{2}H_3$]methionine was achieved after 5 h during both infusion modes.

Methionine plateau was defined as the mean plasma enrichment between 5 and 6 h. Steady-state of [1-^{13}C]homocysteine was not achieved after6hofinfusion (data not shown), and these values were not used to estimate the tissue intracellular precursor enrichment for whole-body fluxes as done previously (28). However, the plasma [1-^{13}C]homocysteine enrichments were used to calculate methionine kinetics in the GIT, because the assumption of steady-state is not necessary. Whole-body methionine kinetics are depicted in Table 1, and rates are expressed as μmol/(kg·h). Whole-body methionine transmethylation, remethylation, and incorporation into protein synthesis were significantly ($p < 0.05$) higher during ID than IV tracer infusion, indicating first-pass splanchnic metabolism. Whole-body transmethylation represented 25% and 27% of methionine flux when infused IV and ID, respectively. Whole-body transsulfuration represented 21% and 20% of methionine flux when infused IV and ID, respectively, and was not significantly different between the infusion groups. Most of whole-

body methionine flux (~80%) was used for protein synthesis and only ~20% was metabolized through transmethylation and transsulfuration.

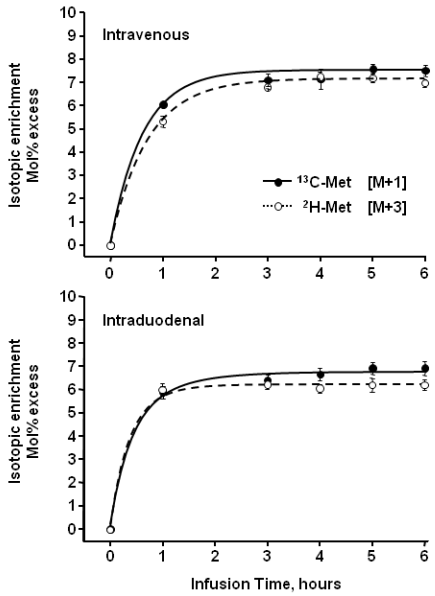

Figure 1. Time course of mean plasma tracer enrichments during IV (upper) and ID (lower) infusion of [1-^{13}C]methionine (filled circles) and [methyl-$^{2}H_3$]methionine (open circles). Enrichments expressed as mole percent excess (MPE). Mean ± SEM (n = 16).

Table 1. Whole-body methionine kinetics.

	IV	ID
[methyl-$^{2}H_3$]methionine flux	130 ± 4	147 ± 2 *
[1-^{13}C]methionine flux	122 ± 3	133 ± 2 *
Transmethylation	33 ± 3	40 ± 3 †
Transsulfuration	26 ± 2	26 ± 2
Remethylation	8 ± 1	14 ± 1 *
Protein Synthesis	97 ± 3	107 ± 2 †

Data are mean ± SEM (n = 16). Rates are expressed as μmol/(kg·h) for IV and ID tracer administration. *P < 0.01 IV vs. ID. †P < 0.05 IV vs. ID

Methionine metabolism in the GIT

Methionine kinetics of the GIT are shown in Table 2 and Figure 2. Total net methionine utilization by the GIT accounted for 20% of the dietary methionine intake. However, there was no first-pass metabolism of dietary methionine in the GIT. After correction for recycling and arterial uptake of ID methionine tracer absorbed into the portal blood, the net methionine uptake was 16 μmol/(kg·h), indicating that the GIT preferentially metabolized systemic methionine rather than dietary methionine. Release of [^{13}C]homocysteine and $^{13}CO_2$ by the GIT, which is a reflection of transmethylation, accounted for 67% of the GIT

[^{13}C]-methionine uptake. Transsulfuration was measured as the $^{13}CO_2$ release by the portal drained viscera, which was 38% of the [^{13}C]methionine uptake by the GIT. The contribution of GIT to whole-body metabolism is shown in Figure 3. The rate of GIT methionine uptake represented 20% of the dietary methionine intake, whereas ~49% and 32% of the dietary methionine intake was metabolized in the whole body via transmethylation and transsulfuration, respectively. The GIT represented 27% and 23% of whole-body rates of transmethylation and transsulfuration, respectively.

Table 2. Steady-state methionine and homocysteine concentrations and isotopic enrichments in plasma and tissue.

	IV	ID
Arterial methionine		
Concentration, μM	202 ± 9	200 ± 8
[1-^{13}C]methionine, MPE	7.56 ± 0.19	6.94 ± 0.09
[methyl-$^{2}H_3$]methionine, MPE	7.05 ± 0.17	6.25 ± 0.08
Portal methionine		
Concentration, μM	208 ± 16	210 ± 17
[1-^{13}C]methionine, MPE	6.37 ± 0.16	7.75 ± 0.24
[methyl-$^{2}H_3$]methionine, MPE	5.97 ± 0.15	7.19 ± 0.22
Arterial homocysteine		
Concentration, μM	7.80 ± 0.91	6.54 ± 0.48
[1-^{13}C]homocysteine, MPE	5.60 ± 0.19	5.52 ± 0.11
Portal homocysteine		
Concentration, μM	8.48 ± 0.76	8.16 ± 0.57
[1-^{13}C]homocysteine, MPE	5.58 ± 0.19	5.63 ± 0.10
Intestine [1-^{13}C]methionine, MPE	3.25 ± 0.29	3.07 ± 0.48
Intestine [methyl-$^{2}H_3$]methionine, MPE	0.34 ± 0.03	0.38 ± 0.09
Liver [1-^{13}C]methionine, MPE	4.34 ± 0.16	3.79 ± 0.16
Liver [methyl-$^{2}H_3$]methionine, MPE	0.24 ± 0.01	0.20 ± 0.01

Data are mean ± SEM (n = 16 plasma and n = 6–8 tissues) for IV and ID tracer administration.

The isotopic enrichments of free [1-^{13}C] and [methyl-$^{2}H_3$]methionine in the intestinal and liver tissues were not different between IV and ID groups (Table 2). However, the enrichment of [$^{2}H_3$]methionine was significantly ($p < 0.01$) lower than [13C]methionine in both the intestine (ratio [$^{2}H_3$]methionine/[^{13}C]methionine = 0.12) and liver (ratio [$^{2}H_3$]methionine/[^{13}C]-methionine = 0.05); the ratio of [$^{2}H_3$]methionine/[^{13}C]methionine was significantly ($p < 0.001$) lower in the liver than intestine. This relationship also was observed for protein-bound [^{13}C]-methionine and [$^{2}H_3$]methionine in the intestinal and liver tissue (data not shown). The net portal fluxes of methionine, homocysteine, and CO_2 are shown in Table 3. Consistent with tracer flux, the net rate of methionine absorption from the GIT was 78% and 82% of the

dietary intake in IV and ID groups, respectively. The net homocysteine flux was significantly positive, indicating net production by the GIT; the homocysteine flux represented 20–25% of the respective GIT methionine utilization rate.

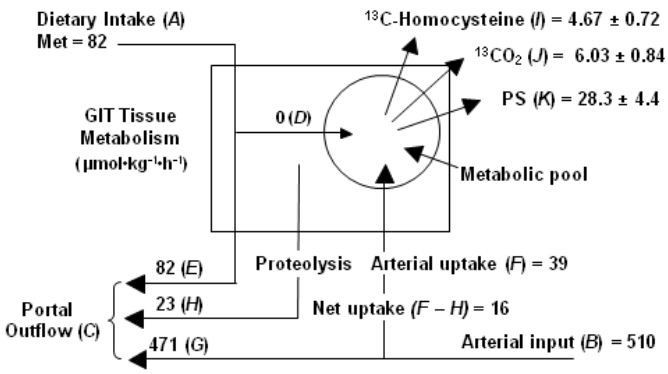

Figure 2. Schematic representation of the metabolic fate of enteral and systemic methionine kinetics in the GIT (n = 16). Dietary methionine intake (*A*); arterial methionine flux through the GIT (*B*); portal methionine outflow (*C*); unidirectional, first-pass uptake of dietary methionine by the GIT (*D*); dietary methionine not metabolized by the GIT in first pass (*E*); unidirectional uptake of arterial methionine by the GIT (*F*); arterial methionine not metabolized by the GIT (*G*); recycled methionine derived from proteolysis (*H*); and methionine that is converted to homocysteine (*I*), CO_2 (*J*), and protein (*K*) by the GIT. Rates expressed as μmol/(kg·h). Dietary intake includes [1-^{13}C] and [methyl-$^{2}H_3$]methionine tracers. Detailed explanation of equations and assumptions used for calculating the methionine kinetic in the GIT are included in supporting information (SI) *Appendix*.

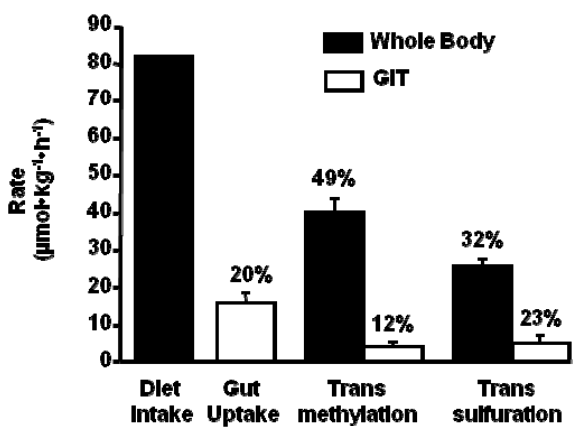

Figure 3. The absolute rates and relative proportions of dietary methionine intake, transmethylation, and transsulfuration in the whole body (filled bars) and GIT (open bars). Rates expressed as μmol/(kg·h). Percentages above filled bars represent whole-body rates relative to dietary methionine intake, whereas those above open bars represent GIT rates relative to the respective whole-body rates. Mean ± SE M(n = 16).

The mRNA expression and activity of CBS in various GI tissues are expressed in Figure 4. A single PCR product of 76 bp derived from a sequence spanning exons 6 and 7 of the porcine CBS gene was measured in all tissues (Figure 4A). The relative expression of CBS mRNA in

liver tissue was ~10-fold higher than in any GI tissue measured. However, the CBS activity in liver tissue was only 3-to 5-fold higher than in GITs. Interestingly, both liver and intestinal epithelial cell CBS activity was markedly (~6-fold) increased above the basal rate by coincubation with 1.0 mM SAM, a known allosteric activator of the enzyme (28).

Table 3. Dietary methionine intake and net portal fluxes of methionine, homocysteine, and CO_2.

	IV	ID
Enteral methionine intake	62	82
Portal plasma flow (liter/(kg·h)	2.58 ± 0.16	2.44 ± 0.12
Net portal methionine absorption	48 ± 3	67 ± 3
Methionine intake, %	78 ± 4	82 ± 3
Net portal methionine utilization	14 ± 3	15 ± 3
Methionine intake, %	22 ± 4	18 ± 3
Net portal homocysteine release	2.7 ± 0.5	3.9 ± 0.5
Portal methionine uptake, %	20 ± 3	25 ± 4
Net portal CO_2 release	6,948 ± 226	6,725 ± 204

Data are mean ± SEM (n = 16). Rates are expressed as µmol/(kg·d) except where indicated.

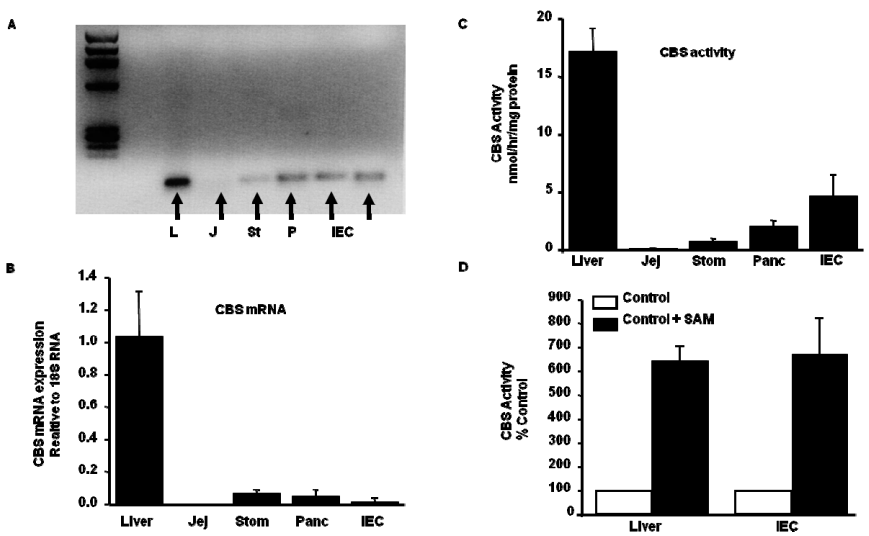

Figure 4. CBS mRNA abundance and enzymatic activity in liver and selected GITs. (*A*) The relative amounts of CBS mRNA product derived from 100 ng of RNA of representative tissue samples from liver (L), jejunum (J), stomach (St), pancreas (P), and intestinal epithelial cells (IEC) after RT-PCR and separation on agarose gel. (*B*) Abundance of CBS mRNA expressed relative to 18S RNA in liver, jejunum, stomach, pancreas, and IEC (n = 4). (*C*) CBS enzymatic activity determined in liver, jejunum, stomach, pancreas, and IEC (n = 4). (*D*) Relative changes in CBS enzymatic activity determined in liver tissue and intestinal epithelial cells in the absence and presence of 1.0 mM SAM (*n* = 3). Mean ± SEM.

DISCUSSION

The main objective of this study was to quantify the metabolic fate of methionine in the GIT by using our established in vivo model coupling organ balance and isotopic tracer kinetics. Our results demonstrate in vivo that the GIT is a metabolically significant site of methionine transmethylation and transsulfuration. Moreover, we show in vivo evidence that the GIT is not only a site of intracellular homocysteine synthesis but also of net release into the circulation. This finding provides evidence for the metabolic role of the GIT in the etiology of homocysteinemia and how this may be impacted by gastrointestinal disease states. This study also extends our previous work with other indispensable amino acids, e.g., lysine, leucine, threonine, and phenylalanine, and shows that, in addition to the liver, the GIT is an active site of methionine metabolism in the body (24-26, 29, 30).

Our results indicated that the GIT metabolize ~20% of the dietary methionine intake. Recent studies in neonatal piglets fed cysteine-free diets demonstrated that the whole-body methionine requirement is ~40% lower in total parenterally fed (TPN) compared with enterally fed pigs (31). The difference in this case was attributed to GIT metabolism, because TPN bypasses the gut and results in gut atrophy. A subsequent study showed that feeding an excess of dietary cystine significantly decreased the methionine requirement consistent with the methionine-sparing effect of cysteine (22). However, even with excess dietary cystine, the methionine requirement in TPN-fed pigs was still ~28% lower than enterally fed pigs, again implying that the GIT represents 28% of body methionine metabolism. The latter study is comparable to our current study where dietary cystine was in excess of the methionine intake. The difference between our direct estimate of 20% and the indirect estimate of 28% GIT metabolism could be due to the nature and composition of the diets (formula versus elemental diet) or that our pigs were slightly older (4 *versus* 1 wk old).

The finding of significant transmethylation and transsulfuration in the GIT is supported by previous reports and our current evidence showing the presence of enzymes involved in methionine metabolism in stomach, pancreas, and intestinal epithelial cells (18, 32). Recent evidence from colonic cell (Caco-2) culture studies also showed the presence of methionine transmethylation and transsulfuration activity (33). The finding of net homocysteine production potentially implicates the gut in homocysteinemia. It also explains why enteral nutrition results in significantly higher circulating homocysteine concentrations than TPN (23). A striking observation was the significant reduction in the tissue-free enrichment of [$^{2}H_{3}$]methionine compared with [^{13}C]methionine; the mean ratio of [$^{2}H_{3}$]methionine/[^{13}C]-methionine was 0.05 in liver and 0.12 in intestine. These ratios are explained by the high rate of intracellular methylation and necessary loss of the deuterated methyl moiety via SAM synthesis. This indicates a substantial methylation rate in the intestine that is nearly half of that observed in the liver. It is important to note that the current estimates of methionine transmethylation and transsulfuration are minimal estimates, given the generous dietary cystine intake, and are likely higher under conditions of cysteine and folate deficiency or oxidant stress. Yet, it is remarkable that despite the high dietary cysteine intake, the GIT represent ~23% of whole-body transsulfuration and a third of all methionine used by the gut

was used for cysteine synthesis. This value is nearly twice as high as that reported recently for sheep, where they observed that ~10% of whole-body methionine oxidation occurred in GIT (27). It is of interest that isolated epithelial cells had the highest CBS activity among the GI tissues measured. Oxidant stress is known to increase methionine transsulfuration to meet the increased cysteine demand for cellular glutathione synthesis (34, 35). This suggests that the metabolic requirement for methionine and cysteine by the gut may be increased in conditions such as inflammatory bowel disease and enteric infection.

We observed substantially higher whole-body rates of transmethylation (3-fold) and transsulfuration (6-fold) in our young pigs when compared with those reported in adult humans (21). Moreover, when expressed as fraction of transmethylation, we found that most of the body homocysteine produced in young pigs is metabolized via transsulfuration, and less is remethylated when compared with adult humans; it is unclear whether this is due to difference in species or age. A particularly important finding was that the whole-body transmethylation and remethylation rates were significantly higher during the enteral compared with the IV tracer infusion protocol. This suggests that nearly 18% and 43% of the whole-body transmethylation and remethylation, respectively, occurs in first-pass by splanchnic tissues. However, another key observation from this study was that the GIT preferentially utilizes methionine from the arterial circulation rather than the diet, contrary to our hypothesis. Thus, the results indicate that the preferential first-pass splanchnic metabolism of dietary methionine occurs in the liver and not the GIT. These findings are consistent with our evidence of substantial intracellular methylation rates in both the liver and intestinal tissues. Moreover, our findings support indirect evidence from separate clinical studies where a dual-labeled [1-^{13}C-methyl-$^{2}H_3$]methionine tracer was given intravenously and orally (20, 21). The authors of these two studies estimated that there is no first-pass splanchnic metabolism when based solely on kinetics of the [1-^{13}C-methyl-$^{2}H_3$]methionine isotopomer. However, these authors found that the enrichment of [1-^{13}C]methionine isotopomer was considerably higher when the [1-^{13}C-methyl-$^{2}H_3$]methionine tracer was given orally than after IV infusion, indicating the presence of significant first-pass splanchnic transmethylation and remethylation of dietary methionine. Recent in vivo studies in sheep also indicate that the liver and gut tissues are important sites of homocysteine remethylation (36). The markedly lower enrichment of the [methyl-$^{2}H_3$]methionine compared with [1-^{13}C]-methionine implies a very high fractional rate of methylation and remethylation. Therefore, our results together with previous reports in humans and sheep strongly suggest that the GIT and liver are major sites of homocysteine remethylation.

In this study, the relative plasma [^{13}C]homocysteine/[^{13}C]methionine enrichments were considerably higher than recent reports in humans under post-absorptive (37, 38) and insulin stimulated conditions. Theoretically, in tissues with the enzymatic capacity for transmethylation the intracellular enrichment of [^{13}C]methionine and [^{13}C]homocysteine should be similar, assuming that there is one common intracellular pool. Although we did not measure tissue [^{13}C]homocysteine enrichments, the tissue [^{13}C]methionine enrichment in the intestine (3.07 MPE) and liver (3.79 MPE) was significantly lower than that of plasma [^{13}C]-homocysteine (5.52 MPE). The plasma [^{13}C]homocysteine enrichment theoretically represents

the summation of transmethylation in the whole body. These results further suggest that the fractional rates of methionine transmethylation to homocysteine are disproportionately higher in the liver and GIT compared with other tissues in the body.

In conclusion, this study provides in vivo kinetic evidence for methionine transmethylation and transsulfuration in the developing GIT. Moreover, we show the GIT to be a site of net homocysteine release into the circulation. The high rates of transmethylation in the GIT may be functionally linked to methyl demand for synthesis of polyamines, phosphatidylcholine, or creatine (39). The GIT consumed 20% of the dietary methionine intake and approximately two-thirds of this was used for synthesis of homocysteine and cysteine. We also show that the GIT contributes significantly to whole-body methionine transsulfuration and that CBS expression is localized to epithelial cells within the small-intestinal mucosa. We speculate that the high rate of methionine transsulfuration in the GIT is driven by cysteine needs for glutathione synthesis because of the oxidant stress associated with the high metabolic activity of proliferating epithelial cells. Another demand for intestinal methionine transsulfuration may be for synthesis of cysteine-rich mucins secreted by goblet cells involved in innate immune function (40). We also found significant first-pass splanchnic transmethylation and remethylation, but this appears to be confined only to the liver, because there was no first-pass methionine use by the GIT. Taken together, the results suggest that the GIT is a key site for regulation of homocysteine metabolism via dietary and other means. These studies also suggest that GI disease states may play a role in the development and treatment of hyperhomocysteinemia or conversely that dysregulation of gut homocysteine metabolism may contribute to pathologies of GI diseases, namely inflammatory bowel disease.

METHODS

Animals and design

The protocol was approved by the Animal Protocol Review Committee of Baylor College of Medicine and conformed to the U.S. Department of Agriculture guidelines. Sixteen, 14-day-old female piglets (Large White x Hampshire x Duroc) were adapted for 7 days to a liquid milk replacer diet (Litterlife; Merrick, Middleton, WI) that provided 25 g of lactose, 5 g of fat, and 12.5 g of protein per kg body weight daily. Methionine and cystine contents were 0.52% and 0.61%, respectively, providing a daily intake of 0.25 g methionine/kg and 0.31 g of cystine/kg. At 20 d of age, the piglets (n = 16) were surgically implanted with catheters as described (26, 41). One week after surgery, each piglet received ID and IV tracer infusions on two different days in a randomized, cross-over design with at least one day between the ID and IV infusions. To estimate whole-body CO_2 production, a 2-h infusion of [^{13}C]sodium bicarbonate was performed before the methionine tracer infusion (26).

Tracer Protocol

At 27 days of age, piglets were fed a bolus meal supplying 1/12 of the preceding total intake

after an overnight fast. Constant duodenal infusion at a rate of 1/24 of the daily intake (ml/(kg·h)) was started 1h after the animal had consumed its meal; this was continued for 8 h. Immediately after starting the duodenal feeding, all piglets received a primed, continuous IV infusion (5 and 10 µmol/(kg·h), respectively) of [^{13}C]sodium bicarbonate (99% APE; Cambridge Isotope Laboratories, Andover, MA) for 2 h. At 120 min, the [^{13}C]bicarbonate infusion ended, and an immediate infusion of [1-^{13}C]methionine and [methyl-$^{2}H_3$]methionine (98% and 99% APE, respectively; Cambridge Isotope Laboratories) was administrated for 6 h either via the jugular (IV) or via the duodenal (ID) catheter. The primed, continuous infusion rate was 10 µmol/(kg·h) for each methionine tracer, so the total isotopic methionine infusion rate was 20 µmol/(kg·h). Arterial and portal blood samples were collected at 0, 90, 115, and 120 min and 6, 7, and 8 h. Portal blood flow was monitored continuously by transit-time ultrasound for 30 min before the initial formula feeding and throughout the entire 8-h protocol as described (24, 26). After tracer infusion on the second study day, animals were euthanized (Beutanasia-D; Schering-Plough Animal Health, Kenilworth, NJ) and intestinal, pancreas, stomach, and liver tissues were collected and frozen at -80°C.

Sample preparation and analysis

Blood gases (Chiron Diagnostics, Halstead, Essex, U.K.) and hematocrit were determined immediately in all arterial and portal blood samples, and isotopic enrichment of $^{13}CO_2$ in whole blood was analyzed by isotope ratio mass spectrometer as described (26). Plasma was obtained from arterial and portal blood, and an aliquot of plasma was combined with methionine sulfone (0.4 mM) and stored at -80°C until further amino acid analysis by HPLC (PicoTag System; Waters, Woburn, MA).

Measurement of stable isotope tracer enrichments

Plasma isotopic enrichments of [1-^{13}C]methionine, [methyl-$^{2}H_3$]methionine, and [1-^{13}C]-homocysteine were quantified on the heptafluorobutyric anhydride (HFBA) derivatives by GC-MS by using a modification of the methods described (37, 42). Blood $^{13}CO_2$ enrichment was determined by gas isotope ratio mass spectrometry (26). Plasma homocysteine was determined by isotope dilution using D_8-homocystine (37, 42). Briefly, plasma was acidified with 10% trichloroacetic acid, amino acids were separated by cation-exchange (AG 50W-X8; Bio-Rad, Richmond, CA), treated with DTT (DTT; Sigma–Aldrich, St. Louis, MO), and derivatized with HFBA (42). GC-MS was performed by negative chemical ionization on a GC-MS model HP-6890/5973 MSD (Hewlett– Packard, Palo Alto, CA). Ion abundance was monitored with selected-ion monitoring (SIM); mass-to-charge (m/z) for natural methionine [M + 0], [1-^{13}C]methionine [M + 1], and [methyl-$^{2}H_3$]methionine [M + 3] were 367, 368, and 370, respectively. Ions were monitored by SIM at m/z 449–553 for natural homocysteine, [1-^{13}C]homocysteine and D_4-homocysteine. Isotopic enrichments of both tracers were expressed as mole percent excess (MPE), and the concentration of homocysteine was calculated by isotope dilution as described (37). The frozen small intestine (n = 16) and a liver sample were homogenized and separated into free and bound fractions by adding sulfosalicylic acid (6%) to precipitate protein. The tissue-free fraction was then subjected to

cation exchange as described above, the eluant was dried under nitrogen, and derivatized by using ethylchloroformate. GC-MS analysis (Carlo Erba GC800 and Fisons MD800; Interscience BV, Breda, The Netherlands) was performed in electron-impact mode, and the ion abundance was monitored in SIM mode; m/z for natural methionine [M + 0], [1-^{13}C]methionine [M + 1], and [$^{2}H_3$]methionine [M + 3] were 249, 250, and 252, respectively.

CBS measurement

Total RNA was isolated from the frozen porcine liver, jejunum, stomach, and pancreas samples and isolated jejunum cells by using RNeasy Mini kit (Qiagen, Valencia, CA). Approximately 100 ng of total RNA was used for each real-time qRT-PCR. Primers and probe designed from the porcine CBS mRNA (GenBank accession no. BP446783) were as follows: CBS forward primer (200 nM): CAGCGCTGCGTGGTGAT; CBS reverse primer (200 nM): CAGACTCTGTGCGCAACTACATGTCCAAG; and CBS TaqMan TAMRA probe (100 nM): 6FAM-GCAGCATCCACTTGTCACTCA-TAMRA. Primers and probe of ribosomal RNA (18S rRNA; Applied Biosystems, Foster City, CA) were used as an internal control. Assays were performed in triplicate with an ABI Prism 7900 sequence detector (Applied Biosystems). Data were normalized to 18S ribosomal RNA (ΔΔCt analysis). CBS activity was determined as described (18, 43). Separate reactions were carried out in the presence and absence of 1.0 mM SAM to test for allosteric induction of CBS.

Calculations

We quantified whole-body and GIT methionine, homocysteine, and CO_2 kinetics as described (26, 37, 44) (see *SI Appendix*).

Statistics

Data are expressed as mean ± SEM. Differences in whole-body methionine kinetics between IV and ID infusions were analyzed by paired *t* test. Tissue samples were analyzed by paired *t* test and nonparametric Mann–Whitney test. A value of $p < 0.05$ was considered to be statistically significant. All statistical analyses were performed by using SPSS (version 11.0; SPSS Inc, Chicago, IL).

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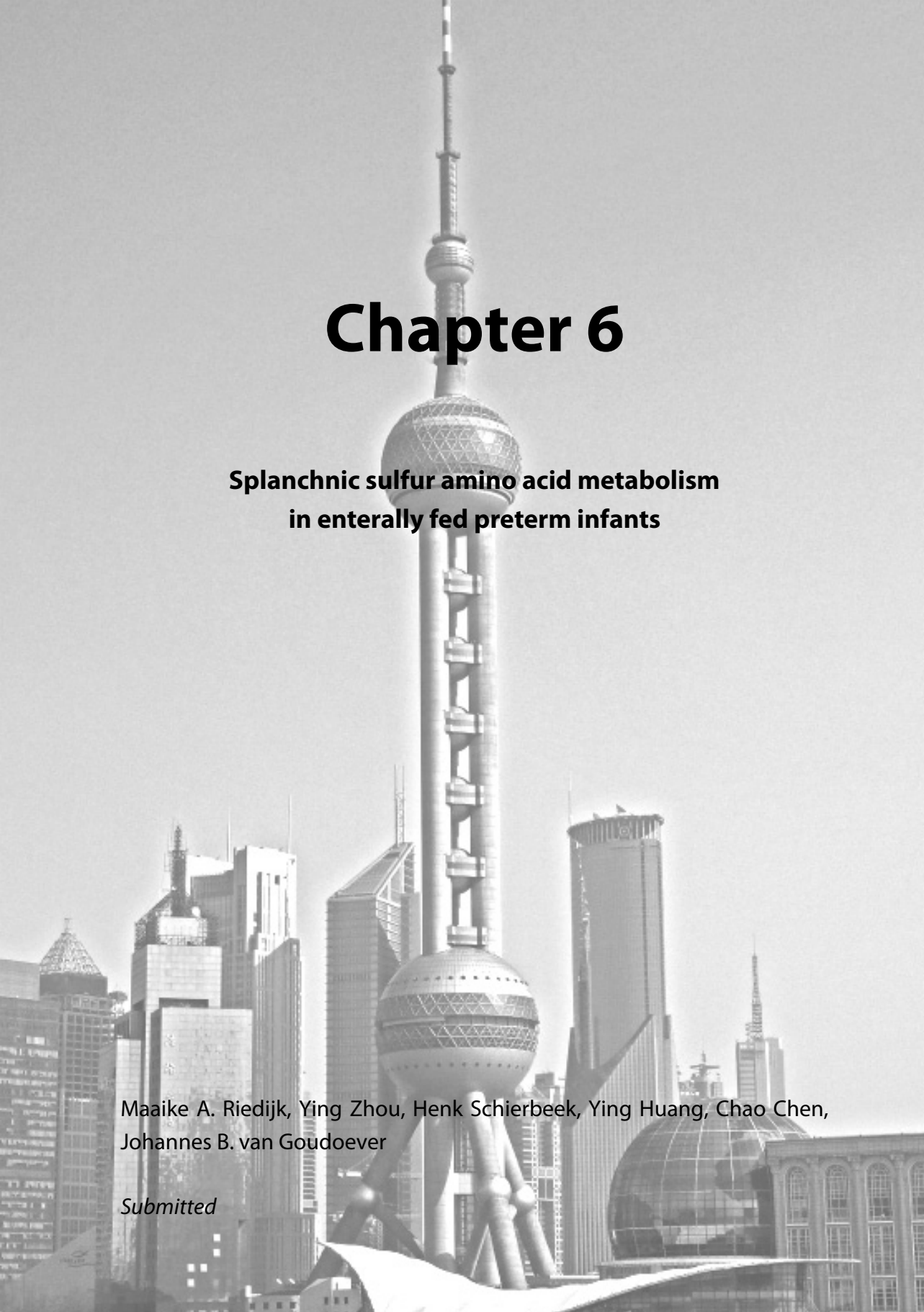

Chapter 6

Splanchnic sulfur amino acid metabolism in enterally fed preterm infants

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Submitted

ABSTRACT

Methionine is the only indispensable sulfur-containing amino acid and acts as a precursor for the dispensable sulfur amino acids homocysteine, cysteine and taurine. In preterm infants, however, cysteine is believed to be a conditionally indispensable amino acid as their enzymatic pathways involved in cysteine synthesis are still immature. The gastrointestinal tract plays a crucial role in methionine metabolism in newborn piglets and adult humans. Completely unexplored, however, is the possible role of the splanchnic tissues of human preterm infants in methionine metabolism, where cysteine synthesis could occur.

Using tracer techniques we determined first-pass splanchnic methionine uptake in preterm infants and examined in vivo cysteine synthesis by incorporation of enteral serine in cysteine. Plasma methionine concentration was unchanged after 24 h of cysteine-free feeding, whereas the cystine concentration had decreased significantly. Fractional splanchnic first-pass methionine uptake accounted for 35 ± 15%. Surprisingly, cysteine was not synthesized de novo in first-pass. Thus, plasma cysteine concentration decreased in preterm infants after a period of cysteine-free feeding and splanchnic tissues do not use dietary serine for endogenous cysteine synthesis. These results might indicate that cysteine might be a conditionally essential amino acid in these infants.

INTRODUCTION

Sulfur amino acids (SAA) have attracted much attention in the past few years mainly because of the link to cardiovascular disease (1) and neurological diseases (eg, Alzheimer's disease) (2, 3). The SAA in humans are methionine, homocysteine and cysteine. Homocysteine was found to play an important role in pregnancy complications as well (4) and methionine in neural dysfunction (5).

Methionine is the only indispensable amino acid and serves as a precursor for homocysteine and cysteine. The latter are, therefore, dispensable amino acids. Only methionine and cysteine are proteinogenic. Homocysteine acts as a key intracellular intermediary in the regulation of plasma methionine and cysteine concentrations. Methionine is crucial in several methylation processes, and its sulfur is indispensable to synthesize cysteine. Cysteine is a precursor for glutathione, which is a major intracellular antioxidant, as well as for CoA and taurine.

Homocysteine formed from methionine can either be converted back to methionine or catabolized in the transsulfuration pathway by forming cysteine (Figure 1). In the latter pathway, homocysteine is condensed with serine to form cystathionine, which is regulated by the enzyme cystathionine ß-synthase (CBS, E.C. 4.2.1.22) in the liver. Subsequently, cystathionine is cleaved into α-ketobutyrate and cysteine by cystathionine γ-lyase (E.C. 4.4.1.1), whereby the serine carbon skeleton and the sulfur moiety of homocysteine together form cysteine.

Cysteine is believed to be a conditionally indispensable amino acid in preterm infants in view of their biochemical immaturity (6). Several in vitro studies showed that CBS activity was limited or even absent in liver tissues of deceased preterm infants as compared with term infants (7-9). In vivo studies have reported that the transsulfuration pathway is certainly active and sufficient in very-low-birth-weight infants (10-12). In addition, Stegink and Den Besten (13) showed that besides the liver, the intestine might be important in methionine metabolism. This finding suggests an important role for the gut in cysteine production, which was confirmed in animal experiments (14). Recently, we have demonstrated in formula fed neonatal piglets that the gastrointestinal tissues are indeed a significant site for homocysteine and cysteine production (15). Yet we were not able to establish splanchnic first pass uptake of dietary methionine. As the piglets received a formula containing high cysteine concentrations, first-pass methionine metabolism could have gone unnoticed as a result of the methionine sparing capacity of cysteine, as described in animals and humans (16, 17).

Preterm infants might not be capable of synthesizing large amounts of cysteine de novo. In the present study we therefore determined the splanchnic methionine first-pass uptake in preterm infants fed a cyst(e)ine free diet. After 24-h adaptation, simultaneous enteral ([U-^{13}C]methionine and [1-^{13}C]serine) and intravenous ([1-^{13}C]methionine tracer infusions were performed to determine splanchnic methionine kinetics and to detect the serine carbon label in cysteine.

METHODS

Subjects and study formula

The study protocol was approved by the Central Committee on Research Involving Human Subjects, the Erasmus MC institutional review board and the Children's Hospital Ethics Committee of Fudan University. Written and informed consent was obtained from parents of each subject.

Patients eligible for the study were preterm infants born at gestational age (GA) < 37 wk with a birth weight < 2000 g, and admitted to the neonatal intensive care unit of the Children's Hospital of Fudan University (Shanghai, China P.R.) in the first days of life. Infants needed to be clinically stable and those with any congenital, gastrointestinal or metabolic disease were excluded. Sufficient growth was ascertained from > 10 g/(kg·d) weight gain for at least four days.

Infants were enrolled in the study when they tolerated ≥ 120 mL enteral feeding/(kg·d). Those who did not tolerate an enteral intake of 150 mL/(kg·d) received additional glucose intravenously to provide a total intake of 150 mL/(kg·d). We used hydrolyzed, cyst(e)ine free study formula (Xcys/Neocate; Nutricia Nederland BV, Zoetermeer, the Netherlands/SHS International, Liverpool, UK) containing adequate amounts of methionine and vitamins B2 (150 µg/100 mL formula), B6 (130 µg/100 mL formula), B11 (10 µm/100 mL formula) and B12 (0.3 µg/100 mL formula). The formula amino acid profile is shown in (Table 1).

Table 1. Amino acid profile of study formula.

Amino acid	Formula mg/100 ml	Amino acid	Formula mg/100 ml
Kcal/100 ml	96		
Protein g/100 ml	2.73		
L-Alanine	128	L-Phenylalanine	152
L-Arginine	227	L-Serine	149
L-Aspartic acid	211	L-Threonine	167
L-Cystine	0	L-Tryptophan	67
L-Glycine	200	L-Tyrosine	152
L-Histidine	129	L-Valine	218
L-Isoleucine	206	L-Asparagine	0
L-Leucine	335	L-Citrulline	0
L-Lysine	232	L-Carnitine	2
L-Methionine	54	L-Taurine	6
L-Proline	242	L-Glutamine	287

Study design and tracer protocol

The study diet was initiated 24 h before start of the tracer protocol to ensure that subjects could adapt to the study diet and cyst(e)ine intake. Prior to the study, all infants received standard formula for low-birth-weight infants (Prelactogen; Nestlé, China), which provided 70 kcal per 100 mL. The total intake at the start of the study was left unaltered until completion the next day. All infants were bottle-fed every two hours. After 24-h adaptation, subjects simultaneously received enteral and intravenous tracer infusions to study methionine metabolism (Figure 1). Because interim analysis of plasma methionine enrichments showed low enrichments of the enteral tracer, we decided to increase infusion rates of all tracers. All infants simultaneously received enteral ([U-^{13}C]methionine and [1-^{13}C]serine (both sterile pyrogen free, > 99% atom percent excess (APE) and 98.7% APE, respectively; Cambridge Isotopes, Woburn, MA) and intravenous ([1-^{13}C]methionine (sterile pyrogen free, > 99 % APE; Cambridge Isotopes) tracer infusions for 5 h. In the first 5 infants the primed continuous infusion rates of [U-^{13}C]methionine, [1-^{13}C]methionine and [1-^{13}C]serine were 2.1 mmol/(kg·h), 1.9 mmol/(kg·h) and 8.7 µmol/(kg·h), respectively. In the other infants the infusion rates of [U-^{13}C]methionine, [1-^{13}C]methionine and [1-^{13}C]serine were 3.5 mmol/(kg·h), 3.0 mmol/(kg·h) and 14.2 µmol/(kg·h), respectively. One hour before start of the tracer infusions, the feeding regimen was changed into continuous drip-feeding. The enteral tracers were continuously infused together with the study formula by an infusion pump through a nasogastric tube.

Altogether, 3 blood samples were obtained: the first at the start of the infusion protocol and 2 during plateau phase after 270 and 300 min of infusion, respectively. In 6 subjects an additional blood sample was taken prior to the start of study formula. This sample served to compare plasma methionine and cysteine concentrations before initiation of the study formula and after 24 h of cyst(e)ine free feeding. Blood was centrifuged immediately and plasma was stored at -80 °C until shipment to the Netherlands by courier on dry ice. Upon arrival in Rotterdam, the Netherlands, the samples' frozen state was confirmed for all samples, after which they were again stored at -80 °C until analysis.

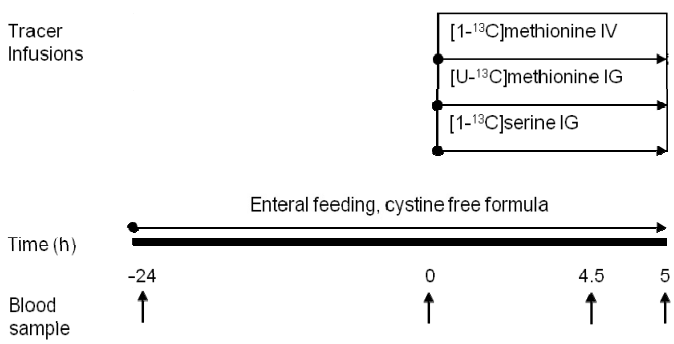

Figure 1. Study design.

Analytical Methods

Plasma amino acid concentrations (mmol/L) were determined by ion exchange chromatography with ninhydrin detection on a Biochrom 20 Amino Acid Analyser (Biochrom

Ltd, Cambridge, England). Plasma isotopic enrichments of [U-^{13}C]methionine, remethylated [$^{13}C_4$]methionine and [1-^{13}C]methionine, [1-^{13}C]serine and [1-^{13}C]cysteine were analyzed by gas chromatography-mass spectrometry (GC-MS). Plasma was treated with 1,4-dithioerythritol (0.2M) (99%, Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature to convert cystine into cysteine. Amino acids were isolated and derivatized using an EZ:Faast (easy-fast amino acid sampling testing) kit (Phenomenex, Torrance, CA, USA). Methionine and cysteine were derivatized to their propoxy-propylester (PCF, Sigma-Aldrich, St. Louis, MO, USA) derivative. Plasma serine enrichment was measured using N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, Sigma-Aldrich, St. Louis, MO, USA) derivative and was incubated overnight at room temperature. Analyses were performed on a Trace gas chromatograph coupled to a Thermo Electron DSQ mass spectrometer (Intersience BV, Breda, the Netherlands). A 30 m x 0.25 mm fused silica capillary column, coated with 0.25 µm VF1701 (Varian, Middelburg, the Netherlands) was used. Samples were measured with selected ion monitoring mode (SIM) and mass to charge (*m/z*) for natural methionine (M+0), [U-$^{13}C_5$]methionine ([M+5), [$^{13}C_4$]methionine (M+4), [1-^{13}C]methionine (M+1) of 277, 282, 281 and 278 respectively. The *m/z* for natural serine (M+0) and [1-^{13}C]-serine (M+1) was 290 and 291, respectively. The *m/z* for natural cysteine (M+0) was 206 and [1-^{13}C]cysteine (M+1) was 207.

Kinetic Analyses and Calculations

The methionine and serine paradigms are illustrated in Figure 2.

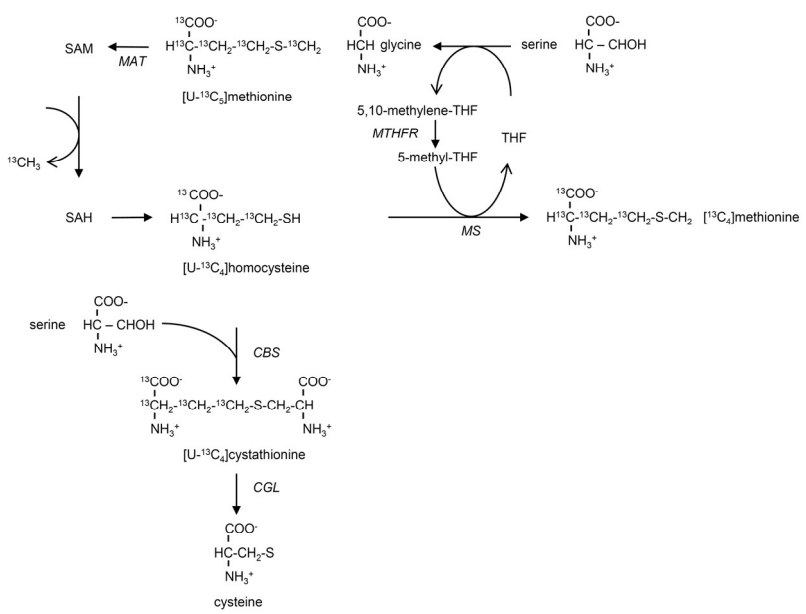

Figure 2. Methionine metabolic pathways. MAT Methionine Adenosyltransferase, SAM S-adenosylmethionine, SAH S-adenosylhomocysteine, CBS Cystathionine ß-synthase, CGL Cystathionine γ-lyase, MS Methionine Synthase, THF Tetrahydrofolate, MTHFR Methylene-tetrahydrofolate Reductase

The enterally administered [U-^{13}C]methionine (M+5) that is transmethylated to homocysteine loses a labeled methyl group, yielding [$^{13}C_4$]homocysteine. This can be metabolized to methionine or be catabolized to form cysteine. Remethylation of [$^{13}C_4$]homocysteine to methionine with an unlabeled methyl group from 5-methyl-tetrahydrofolate results in [$^{13}C_4$]-methionine (M+4). Transsulfuration of [$^{13}C_4$]homocysteine forms [$^{13}C_4$]cystathionine, which is then hydrolyzed to generate unlabeled cysteine. Or [1-^{13}C]serine can be condensed with homocysteine to form cystathionine (M+1), whereby the sulfur moiety is transferred to the carbon skeleton of serine yielding [1-^{13}C]cysteine. Besides, the C_3 of serine may function as a methyl donor in the folate cycle via formation of glycine. (We, therefore, did not use [3-^{13}C]-serine, which forms [1-^{13}C]-methionine that cannot be distinguished from the intravenously infused [1-^{13}C]methionine.) The intravenously administered [1-^{13}C]methionine is metabolized in the methionine cycle but yields unlabeled methionine and cysteine.
We studied amino acid turnover according to Waterlow's stable isotopic dilution model (18). Tracer dilutions were measured during steady state. Whole-body and splanchnic methionine kinetics were calculated using dual stable isotope tracer techniques (19, 20).
Whole-body rate of appearance of enteral methionine was determined by the plasma [U-^{13}C]-methionine enrichment (M+5):

$$Q_{MET\_IG} = i_{MET\_IG} \times [(IE_i/IE_p)-1]$$

where Q_{METIG} is the rate of appearance of enterally infused [U-^{13}C]methionine (µmol/(kg·h), i_{MET_IG} is the enteral methionine infusion rate (µmol/(kg·h) and IE_i and IE_p are the isotopic enrichments (mol% excess of the M+5 enrichment) of the enterally administered [U-^{13}C]-methionine in the infusate and in plasma (M+5) at steady state, respectively.
The rate of appearance of the intravenously administered methionine was based on the following equation:

$$Q_{MET\_IV} = i_{MET\_IV} \times [(IE_i/IE_p)-1]$$

where Q_{MET_IV} is the rate of appearance of intravenously infused [1-^{13}C]methionine (µmol/(kg·h), i_{MET_IV} is the intravenous methionine infusion rate (µmol/(kg·h) and IE_i and IE_p are the isotopic enrichments (mol% excess of the M+1 enrichment) of the intravenously administered [1-^{13}C]methionine in the infusate and in plasma (M+1) at steady state, respectively.
First-pass methionine uptake was calculated as:

$$U = [(Q_{MET\_IG} - Q_{MET\_IV})/Q_{MET\_IG}] \times I$$

where U is the first-pass methionine uptake (µmol/(kg·h), Q_{MET_IG} is the rate of appearance of the enterally administered methionine tracer (µmol/(kg·h), Q_{MET_IV} is the rate of appearance of the intravenously administered methionine tracer (µmol/(kg·h) and I is the intake of dietary methionine (µmol/(kg·h).

The whole-body rate of appearance of serine was calculated as follows:

$$Q_{SER} = i_{SER} \times [(IE_i/IE_p)-1]$$

where Q_{SER} is the rate of appearance of enterally infused [1-^{13}C]serine (μmol/(kg·h), i_{SER} is the serine infusion rate (μmol/(kg·h) and IE_i and IE_p are the isotopic enrichments (mol% excess) of the [1-^{13}C]serine in the infusate and in plasma at steady state, respectively. The plasma [1-^{13}C]serine enrichment was corrected for overestimation of the intracellular [1-^{13}C]serine that occurs when the enrichment of [1-^{13}C]serine in plasma is used. The correction factor (0.4) is derived from adult human in vivo studies (21, 22). As no such correction factor is available for preterm infants, we assumed the same value would be applicable to our subjects.

Statistics

Data are expressed as mean ± SD and are obtained from the samples taken during the last 30 min of the tracer infusions. A value of $p < 0.05$ was considered as statistically significant. Differences between plasma concentrations were analyzed by paired *t* test. Cysteine enrichments comparisons were performed by Student's *t* test. Statistical analyses were performed by using SPSS software (version 14.0; SPSS, Inc, Chicago, IL).

RESULTS

Ten preterm infants were enrolled (Table 2). They were born at mean gestational age 32 ± 2 wk, with a mean birth weight of 1.64 ± 0.16 kg. All infants were clinically stable at the time of the study and were growing > 10 g/day. Three infants received additional glucose intravenously to ensure sufficient caloric intake. The mean total sulfur amino acid intake was 74 ± 9 mg/(kg·d).

Table 2. Patient characteristics.

Patient	Sex	GA wk	SA d	BW kg	SW kg	Kcal kg/d	Methionine mg/(kg·d)
1	M	31	15	1.64	1.70	130	65
2	M	30	26	1.56	1.69	143	80
3	F	32	13	1.95	1.94	125	64
4	M	36	26	1.55	1.91	156	88
5	M	30	16	1.84	1.81	146	82
6	M	33	8	1.60	1.51	127	64
7	F	31	9	1.65	1.58	117	66
8	F	33	11	1.40	1.45	132	67
9	M	32	14	1.74	1.72	141	79
10	M	34	22	1.50	1.65	161	83
Mean	M/F 7/3	32	16	1.64	1.70	138	74
SD		2	7	0.16	0.16	14	9

GA, gestational age; SA, study age; BW, birth weight; SW, study weight.

To define a possible effect of the study formula on the plasma amino acid profile, in 6 infants concentrations of all amino acids were determined prior to the introduction of the study formula and after 24 h of cyst(e)ine-free feeding (Table 3). Mean plasma methionine concentrations of the two feeding regimens did not differ (44 ± 5 *versus* 32 ± 11 μmol/L, $p = 0.08$). In contrast, plasma cystine concentrations differed significantly (37 ± 6 *versus* 19 ± 8 μmol/L, $p = 0.003$). This finding implies that cyst(e)ine consumption exceeded endogenous synthesis.

Table 3. Plasma amino acid profile before the introduction of the study formula (period A) and after 24 h of study formula feeding (period B).

Amino Acid	Period A μmol/L	Period B μmol/L	Reference range (48) μmol/L
Alanine	169 ± 61	217 ± 57	137-362
Arginine	91 ± 17	109 ± 47	11-88
Asparagine	65 ± 21	18 ± 8	16-21
Aspartate	11 ± 1	9 ± 2	5-46
Cystine	37 ± 6	19 ± 8 *	33-55
Glutamate	74 ± 56	70 ± 28	76-551
Glutamine	552 ± 149	384 ± 77	147-623
Glycine	257 ± 45	329 ± 70	66-432
Histidine	65 ± 10	71 ± 15	25-126
Isoleucine	50 ± 8	82 ± 39	31-124
Leucine	88 ± 11	151 ± 72	86-171
Lysine	170 ± 53	186 ± 67	65-282
Methionine	44 ± 5	32 ± 11	21-52
Phenylalanine	62 ± 9	86 ± 17	35-112
Proline	183 ± 50	264 ± 60	
Serine	162 ± 31	176 ± 17	79-227
Taurine	66 ± 11	68 ± 16	
Threonine	316 ± 93	379 ± 110	67-143
Tyrosine	123 ± 35	104 ± 22	48-122
Valine	118 ± 17	163 ± 41	56-154

Data are expressed as mean ± SD (n=6). * Statistically significant $p < 0.05$.

Plasma tracer enrichments after 5 h infusion and tracer kinetics are given in Table 4. Approximately one third of the dietary methionine was taken up by the splanchnic tissues in first-pass metabolism. We investigated the methionine remethylation by determining [$^{13}C_4$]methionine (see also Figure 1). Nevertheless, we were not able to detect enrichment in [$^{13}C_4$]methionine [M+4] by GC-MS, which indicates that under these conditions remethylation is not occurring on a large scale. Since we found a relatively large enrichment in glycine (approximately 1 MPE), enough 5,10-methylene-THF, which contributes to the remethylation, should have been available.

To investigate the endogenous cysteine synthesis we administered labeled serine enterally.

The serine carbon skeleton, together with the sulfur-moiety of homocysteine, is used for cysteine synthesis. We were not able, however, to detect labeled cysteine in plasma by either GC-MS and IRMS. The latter technique is 1000 times more sensitive and, therefore, able to detect enrichments below 0.1 mol % excess (MPE). Surprisingly, as mentioned above, plasma glycine enrichment was approximately 1 MPE, implicating that serine was metabolized to a large extent.

Table 4. Tracer infusion rates, plasma enrichments and kinetics after 5 hours of infusion.

Labeled amino acid	Infusion	Infusion rate	Plateau enrichment	WB Q	First pass uptake
		μmol/(kg·h)	MPE (%)	μmol/(kg·h)	%
[$^{13}C_5$]methionine	IG	2.67 ± 0.70	2.21 ± 0.70	120 ± 26	35 ± 15
[$^{13}C_1$]methionine	IV	2.38 ± 0.57	3.08 ± 0.55	75 ± 15	
[$^{13}C_1$]serine	IG	10.56 ± 2.79	1.42 ± 0.27	727 ± 168	
[$^{13}C_1$]glycine		0	0.97 ± 0.16		
[$^{13}C_1$]cysteine		0	n.d.		

Data are expressed as mean ± SD (n=10). MPE, mol % excess; WB Q, whole body flux.

DISCUSSION

In the present study we described the sulfur amino acid metabolism in enterally fed low-birth-weight infants. We aimed at quantifying the splanchnic first-pass uptake of dietary methionine and observing the endogenous cysteine synthesis by incorporation of enterally administered serine. Plasma cystine concentrations had decreased significantly after a period of cystine-free feeding. Surprisingly, under these conditions enteral administered serine had not been used for endogenous cysteine synthesis.

Splanchnic tissues appear to have a key regulatory role in the distribution of dietary supplied amino acids in neonatal piglets and humans (23-26). The metabolic fates of sequestered dispensable and indispensable amino acid differ widely. For instance, the major metabolic fate of dietary glutamate in the splanchnic region is oxidation (27-29). In contrast, while in both neonatal animals and preterm infants the intestine extracts almost all threonine in first-pass like for glutamate, yet the primary fate is mucosal (glyco-)protein synthesis instead of oxidation (30, 31). First-pass methionine uptake has been examined in both animals and humans. Studies in neonatal piglets showed that 20-50% of the dietary methionine was taken up by the portal drained viscera (liver, intestine, spleen, stomach and pancreas) in first-pass metabolism (24, 32). In agreement with these findings, the total sulfur amino acid requirement for parenterally fed neonatal piglets, as defined by the methionine requirement in absence of dietary cysteine, was 30% lower than for enterally fed piglets (33). The latter finding indicates that also the intestine extracts dietary methionine in first-pass metabolism.

In human adults the splanchnic methionine uptake ranged from 20-33% during the postabsorptive state (34, 35). During the fed state, no first-pass uptake was observed. Different from findings in fed adult humans but similar to findings in fasted adults, we found that 35% of the dietary methionine, in the absence of cystine, was taken up by the splanchnic tissues in the fed state. Furthermore, during enteral administration of [1-^{13}C, methyl-$^{2}H_3$]-methionine Raguso et al. (34) detected more [1-^{13}C]methionine in both fed and fasted state than during intravenous administration. The methionine methyl group is donated in transmethylation and is therefore lost, while the carbon label is retained. Using the dual labeled methionine carries a high likelihood of underestimating first-pass metabolism because methionine is rapidly transmethylated to homocysteine in the gastrointestinal tract by losing the methyl label. Caution should be exercised in using this tracer. We therefore avoided the methyl labeled methionine and used two different labeled carbon tracers which intermediates do not overlap.

We have previously shown that the intestine plays a key role in methionine metabolism (15). Approximately half of all methionine utilized by the gut was metabolized via transmethylation and one third via transsulfuration (cysteine synthesis). Given this knowledge, it is of interest to know the endogenous cysteine synthesis in humans and especially in preterm infants as their enzymatic pathway involving transsulfuration is likely to be still immature. To optimize the transsulfuration pathway, we removed cystine from the diet. We have previously concluded that cysteine is not a conditionally indispensable amino acid in low-birth weight infants (12). Consequently, it was ethically justified to eliminate cysteine from the diet for a short time. Transsulfuration requires vitamin B6 as a cofactor and was therefore adequately supplied according to estimated requirements for preterm infants already in the period before the infants entered the study (36). We did not determine vitamin B6 levels since this requires a large amount of blood, which cannot be drawn from these infants. To study endogenous cyst(e)ine synthesis we have used an established method previously applied in adult humans to study one-carbon metabolism (37-40). However, in these studies plasma cysteine enrichment could not be demonstrated. It is likely that the route of tracer administration plays a role in serine incorporation into cysteine. Stegink and Den Besten (13) demonstrated in adult humans fed a cystine-free diet but otherwise adequate amounts of methionine that plasma cystine concentrations were significantly lower during intravenous feeding than intragastric feeding. Thus, the intestine is an important site for cysteine synthesis. In contrast to previous studies, we therefore administered the serine tracer enterally, to amplify the effect of serine incorporation by the gastrointestinal tract. Nonetheless, like previously described for the intravenously administered labeled serine, we did not find labeled cysteine in plasma by using gas chromatography-mass spectrometry (GC-MS) techniques. To increase sensitivity we analyzed plasma cysteine enrichment again by isotope ratio mass spectrometry (IRMS), which is much more sensitive to detect a labeled carbon atom. Again, no labeled cysteine could be detected. Thus the enteral route of tracer administration did not yield labeled cysteine in plasma. Because approximately 95% of the serine flux is from endogenous synthesis (41), dilution of tracer might be an explanation. Another possible explanation is the limited tracer

infusion time. The infusion period lasted five hours, which could be too short for enteral serine to be incorporated in cysteine. Davis et al. used a nine hour tracer infusion period (39, 40, 42). They also determined the plasma cystathionine enrichment derived from labeled methionine and showed that steady-state was achieved within four hours of tracer infusion. Thus, five hours should suffice to reach a steady-state transsulfuration rate. In addition, the [U-^{13}C]methionine tracer model allows measurement of homocysteine transmethylation rate and remethylation rate by [$^{13}C_4$]homocysteine and [$^{13}C_4$]methionine, respectively. Unfortunately, we were not able to measure homocysteine enrichment in plasma. Surprisingly, we could not detect [$^{13}C_4$]methionine enrichment in plasma. Thus, homocysteine formed by transmethylation was not remethylated back to methionine but was immediately directed to transsulfuration, which yields undetectable unlabeled cysteine. Alternatively, the intestinal cyst(e)ine consumption exceeded the endogenous synthesis. Cysteine is not only needed for glutathione synthesis but also for the production of mucins, the building blocks of the mucus layer that contain large amounts of cysteine. A third possibility is use of cysteine for taurine and sulfate synthesis. We have not determined the presence of label in intestinal glutathione, mucin or taurine.

We found a drop in plasma cystine concentration after a cystine-free feeding period of 24 h, while the plasma methionine concentration remained unchanged. Low plasma cysteine concentration is associated with total parenteral feeding (43-45), compared to plasma concentrations of the breast-fed or formula fed infant (33-55 µmol cystine/L). The initial cystine concentration of 37 ± 6 µmol/L plasma was within the reference range, but decreased significantly to 19 ± 8 µmol/L after 24 h of cystine-free feeding. Yet plasma concentration may not be the best primary endpoint to examine endogenous cysteine synthesis. It is a static measurement that does not provide information about the metabolic fate of an amino acid. Several studies have attempted to increase plasma cyst(e)ine concentration by cysteine supplementation during total parenteral feeding with no effect (46, 47).

In conclusion, in enterally fed low-birth-weight infants at postmenstrual age of 34 wk the splanchnic tissues extract one third of the dietary methionine in first-pass. Under these conditions, enteral serine is not used for endogenous cysteine synthesis. Although recent studies conclude otherwise (10, 12), cysteine synthesis might be limited in preterm infants Further research should be aimed at examining the in vivo transsulfuration pathway in preterm infants.

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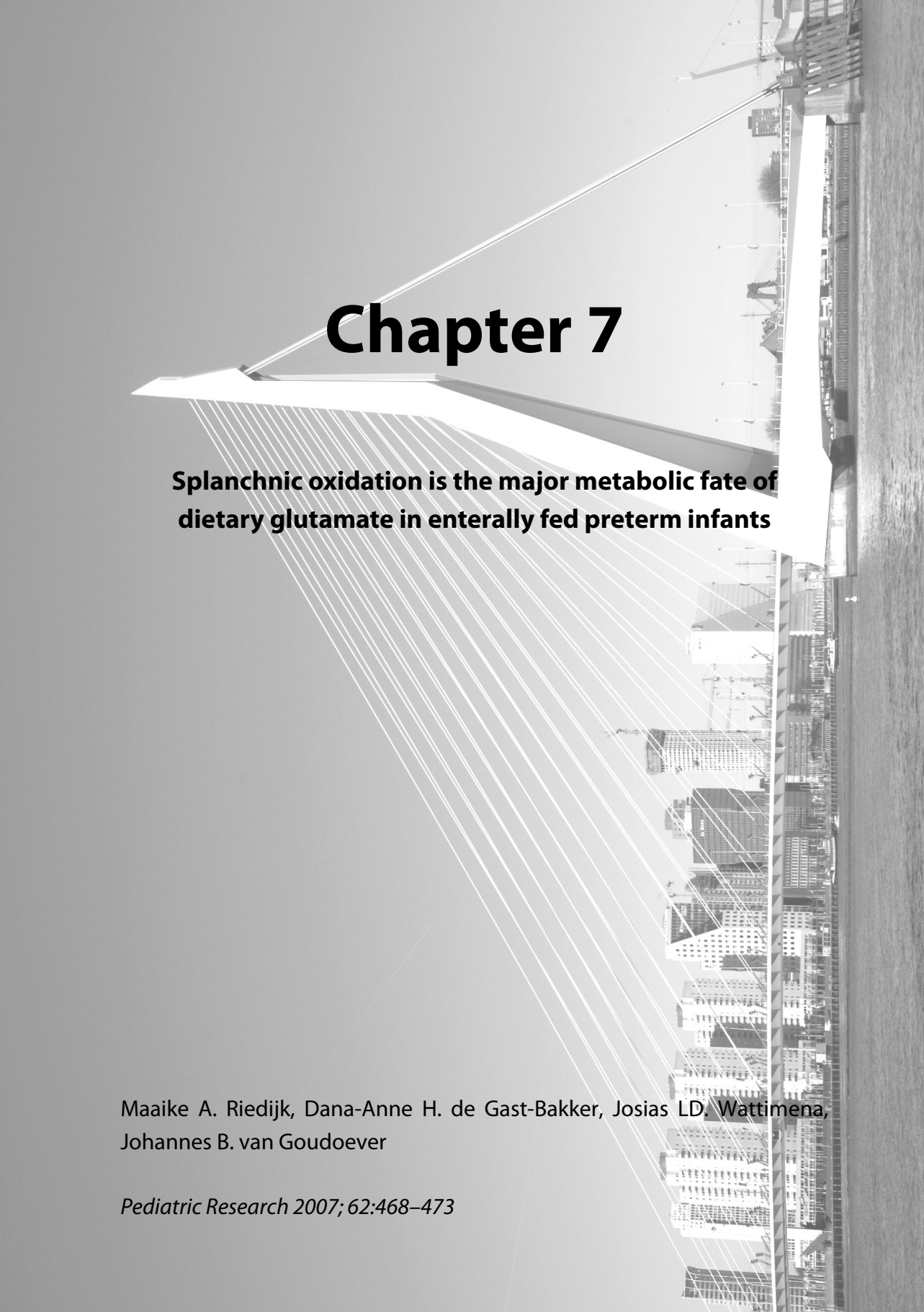
Splanchnic metabolism of dispensable amino acids



Chapter 7

Splanchnic oxidation is the major metabolic fate of dietary glutamate in enterally fed preterm infants

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ABSTRACT

The intestine is a major site of amino acid metabolism, especially in neonates. The energy needed for the metabolic processes in neonatal animals is derived from dietary glucose and amino acids. No data are available showing that dietary amino acids function as intestinal fuel source in human neonates as well.

We hypothesized that preterm infants show a high splanchnic first-pass glutamate metabolism and the primary metabolic fate of glutamate is oxidation.

Five preterm infants (birth weight 1.2 ± 0.2 kg, gestational age 29 ± 1 wk) were studied by dual tracer ([U-^{13}C]glutamate and [D_3]glutamate) techniques on two study days (within postnatal d 14-19). Splanchnic and whole-body glutamate kinetics were assessed by plasma isotopic enrichment of [U-^{13}C]glutamate and [D_3]glutamate and breath $^{13}CO_2$ enrichment.

Fractional first-pass glutamate uptake was 77 ± 18% on d 1 and 70 ± 7% on d 2, mean 74 ± 13%. Almost all (86 ± 7%) of the glutamate used in first-pass is directed toward oxidation.

There is a high splanchnic fractional first-pass uptake and a high oxidation rate of glutamate in preterm infants. Glutamate is an important source of energy for the splanchnic tissues in preterm infants receiving full enteral feeding.

INTRODUCTION

The splanchnic tissues have much higher energy expenditure and protein synthesis rates than expected based on their weight. Studies in neonatal animals found that up to 50% of the dietary protein intake in the portal drained viscera (intestines, pancreas, spleen and stomach) is used for protein turnover and biosynthetic pathways; from 10 to 20% is used for whole-body energy expenditure (1-3). Humans also show considerable intestinal amino acid metabolism (4, 5) and the metabolic rate is even higher in preterm infants. Previous studies showed that first-pass metabolism of leucine in newborns is twice that of adults (6) and that in preterm infants, a great amount of dietary lysine is used in first-pass as well (7).
Glutamate plays a pivotal role in human metabolism. It provides a critical link between carbohydrate and amino acid metabolism. Glutamate is the obligatory precursor of glutamine; it plays a central role in all transamination reactions in the body and also functions as a neurotransmitter. In addition, dietary glutamate is a major substrate for glutathione (GSH) synthesis (8). Animal studies showed that the gut of infant piglets metabolizes virtually all the enteral glutamate during absorption (9) and that glutamate is an important oxidative substrate for the intestinal mucosa (10). In human adults, most of the dietary glutamate (88%) is metabolized in first-pass as well (11) and it is oxidized to a great extent in the splanchnic region (12). These studies in animals and adult humans suggest that dietary glutamate serves as an important metabolic fuel source in the intestine. If so, this may have a particular impact on the nutritional needs of preterm infants. The intestine of preterm infant is immature and has a low tolerance to enteral feeding. Yet, enteral feeding is a must for intestinal maturation and the growth and development of other organs. Much effort should be directed toward understanding the nutritional needs of the intestine of preterm infants. The present study was developed to extend the previously mentioned observations made in animals and adult humans and thus gain more insight into the nutritional needs of infants.
In the present study, we, therefore, investigated 1) the effect of first-pass splanchnic uptake of glutamate and 2) the metabolic fate of the used glutamate in the splanchnic bed of enterally fed preterm infants. Glutamate kinetics in the splanchnic region were studied by dual, primed, constant intravenous (IV) and enteral infusions of amino acids labeled with stable isotopes and preceded by an IV labeled sodium bicarbonate infusion as described before (2, 7). We hypothesized that preterm infants would show significant splanchnic first-pass uptake and oxidation of glutamate.

METHODS

Patients

The Erasmus MC Institutional Review Board approved the study. Written informed consent was obtained from parents or legal guardian. Eligible for this study were preterm infants admitted to the Neonatal Intensive Care Unit of the Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands. Inclusion criteria were birth weight between 750 g and 1500 g,

appropriate for gestational age according to the charts of Usher and Mclean and with CRIB scores < 5 on the first day of life (13). Exclusion criteria were congenital anomalies and gastrointestinal and liver diseases. All enrolled infants received full enteral feeding at the time of the study and were clinically stable. Nenatal (Nutricia Zoetermeer, the Netherlands) was the sole enteral nutrition from 10 h before start of the study and during both study days.

Protocol

To investigate splanchnic and whole-body glutamate kinetics, a crossover study design was applied on two consecutive study days. During the tracer infusion infants were fed continuously through a nasogastric tube. All received the same feeding regimen on both study days. A peripheral IV catheter was available for infusion of tracers during both days. Blood samples were obtained by heel stick. Breath samples were obtained by the direct sampling method as described previously (14, 15). Briefly, in mechanically ventilated neonates a syringe was connected to the ventilator tubing and breath samples were taken slowly during expiration with a total volume of 15 mL. When breathing was spontaneously a 6F gastric tube (6 Ch Argyle; Cherwood Medical, Tullamore, Ireland) was inserted 1 to 1.5 cm into the nasopharynx and end-tidal breath samples were taken slowly with a syringe connected at the end. Collected air was transferred into 10-mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and stored at room temperature until analysis (15).

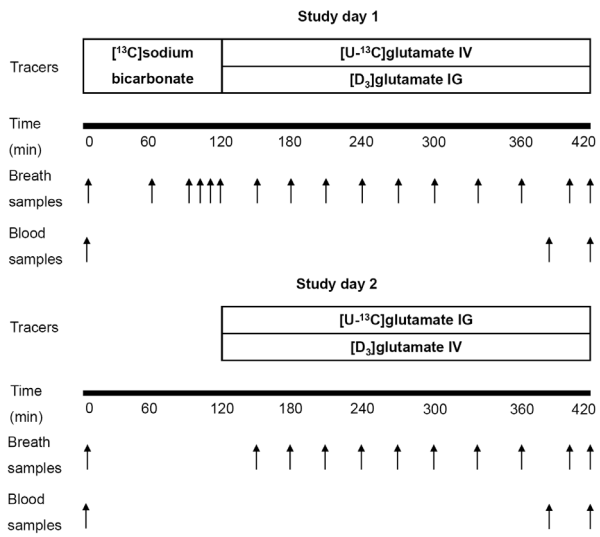

Figure 1. Study design.

The study design is depicted in Figure 1. Three different stable isotopes were infused during study d 1. First, a primed (10 µmol/kg) constant (10 µmol/(kg·h)) IV infusion of [^{13}C]-bicarbonate (99.0 mol% ^{13}C; Cambridge Isotopes, Woburn, Massachusetts, USA) dissolved in sterile saline was administered IV for 2 h. This was immediately followed by primed 5-h infusions (30 µmol/kg and 30 µmol/(kg·h)) of [U-^{13}C]glutamate (95.0 mol% ^{13}C; Cambridge

Isotopes) and (30 µmol/kg and 30 µmol/(kg·h)) of [2,2,4-D_3]glutamate (97.0 mol% D_3; Cambridge Isotopes). [U-^{13}C]glutamate was given via an IV catheter and [D_3]glutamate via the intragastric catheter. On d 2, we switched the IV and intragastric routes. All isotopes were tested and found to be sterile and pyrogen free before use. On each day, baseline blood and breath samples were collected at time 0. During the last half hour of each tracer infusion, breath samples were collected at 15-min intervals and two blood samples were obtained after 4.5- and 5-h tracer infusion. The total amount of blood withdrawn per study day was 1.5 mL, which is less than 2% of the blood volume in a 1000-g infant. Blood was centrifuged immediately and plasma was stored at -80°C for further analysis.

Analytical methods

Plasma enrichments of [U-^{13}C]glutamate and [D_3]glutamate were measured by gas chromatograph-mass spectrometry (GC-MS), using *N*-methyl-*N*-(tert-butyldimethyl) silytrifluoroacetamide (MTBSTFA) derivates. Briefly, 50 µl of plasma was deproteinized with 50 µl 0.24 M sulfosalicylic acid. After centrifugation (for 8 min at 4°C, and 14000x *g*), the supernatant passed over a Dowex cation-exchange resin column (AG 50 W-X8, hydrogen form, Bio-Rad Laboratories, Richmond, CA). The column was washed with 3 mL of water and the amino acids eluted with 1.5 mL 3 M NH_4OH. The eluate was dried at 70°C under nitrogen and derivatives of the amino acids were finally formed by adding 35 µl acetonitrile and 10 µl of MTBSTFA (Pierce, Omnilabo, Breda, the Netherlands) and incubation at 60˚C for 30 min. Analyses were performed on a Carlo Erba GC8000 gaschromatograph coupled to a Fisons MD800 mass spectrometer (Intersience BV, Breda, the Netherlands) by injecting 1 µL with a split ratio of 50:1 on a 25 m x 0.22 mm fused silica capillary column coated with 0.11 µm HT5 (SGE, Victoria, Australia). Ion abundance was monitored by selected ion monitoring and mass to charge for natural glutamate [M+0] was 432 and for D_3-glutamate [M+3] and [U-^{13}C]-glutamate [M+5], 435 and 437, respectively.

$^{13}CO_2$ isotopic enrichment in expired air was measured by isotope ratio mass spectrometry (ABCA; Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands) and expressed as atom percent excess (APE) above baseline. APE was plotted relative to time. Steady state was defined as three or more consecutive points with a slope not different from zero.

Calculations

We studied amino acid turnover according to Waterlow's stable isotopic dilution model (16). Whole-body CO_2 kinetics were calculated as described previously (2). Substrate turnover was calculated by using dual stable isotope tracer techniques. Tracer dilutions were measured during steady state as described previously (7, 17, 18). The equations used to obtain the results are detailed in the Appendix.

Statistics

Data are expressed as mean ± SD values obtained from samples taken over the last hour of each tracer infusion. Differences in background and steady-state enrichments between the two study days were analyzed by paired *t* tests. A value of $p < 0.05$ was considered to be

statistically significant. Statistical analyses were performed by using SPSS software (version 14.0; SPSS Inc, Chicago, IL).

RESULTS

Characteristics of the five enrolled infants are listed in Table 1. Their mean gestational age was 29 ± 1 wk and mean birth weight 1.18 ± 0.23 kg. All were clinically stable at the time of the study. On d 1, four infants needed supplemental oxygen by a nasal prong; on d 2 only two infants needed supplemental oxygen. They all needed caffeine to support breathing, but no other medication was prescribed. Baseline ^{13}C enrichment in expired breath on d 1 (1.0867 ± 0.0005 APE) did not differ from that on d 2 (1.0889 ± 0.0029 APE) ($p = 0.17$) and were in the same range as we have found previously (15). Also, baseline enrichments of [U-^{13}C]glutamate and [D_3]glutamate in plasma did not significantly differ for the two study days.

Table 1. Patient characteristics.

Patient	GA wk	BW kg	SA 1 d	SA 2 d	SW 1 kg	SW 2 kg	Kcal
1	31	1.29	9	11	1.25	1.28	108
2	27	0.92	13	19	0.94	1.12	123
3	29	1.45	15	17	1.46	1.48	106
4	29	1.30	21	22	1.41	1.41	109
5	30	0.96	12	25	0.96	0.99	101
Mean	29	1.18	14	19	1.20	1.26	109
SD	1	0.23	4	5	0.25	0.20	8

GA, gestational age; BW, birth weight ; SA 1, postnatal age at day study d 1; SA 2, postnatal age at study d 2; SW 1, weight at study d 1; SW 2, weight at study d 2; Kcal = kilocalories/(kg·d).

To calculate splanchnic and whole-body glutamate kinetics, we used plateau glutamate enrichment values from plasma (Table 2) and labeled carbon dioxide in expired breath. Although we obtained only two blood samples during the tracer infusions, a steady state must have been reached at the time we drew the plasma samples, because at that time the $^{13}CO_2$ excretion was at isotopic plateau. As shown in Figure 2, steady state of $^{13}CO_2$ during enteral and IV [U-^{13}C]glutamate infusion was achieved within 2 h of infusion. The slope of the line from time point 120 min to 300 min was not different from zero ($p = 0.40$). As expected, plateau enrichment (mole % excess) of the enterally administered tracer was much lower than that of the tracer administered IV.

Table 3 shows the results of whole-body and splanchnic glutamate kinetics. The mean estimated whole-body CO_2 production was 32.3 ± 5.3 mmol/(kg·h). These values are in the same range as we have reported previously (7, 15). The rate of appearance of [U-^{13}C]-glutamate or flux (Q) during IV administration on d 1 was 1287 ± 864 µmol/(kg·h).

Table 2. Steady state isotopic enrichments of [U-^{13}C]glutamate and [D_3]glutamate in plasma on study d 1 and 2

Patient	**[U-^{13}C]glutamate** (mol% excess)					
	Day 1 Δ			Day 2 Δ		
	4.5h	5h	mean	4.5h	5h	mean
1	0.82	0.91	0.87	0.50	-	0.72
2	3.99	4.06	4.01	1.23	1.45	1.61
3	3.19	3.15	3.17	0.44	0.44	0.68
4	1.54	1.77	1.66	0.19	0.15	0.38
5	2.22	2.27	2.25	0.18	0.18	0.50
Mean	2.35	2.43	2.39	0.51	0.56	0.52
SD	1.27	1.22	1.23	0.43	0.61	0.47
Patient	**[D_3]glutamate** (mol% excess)					
	Day 1 Δ			Day 2 Δ		
	4.5h	5h	mean	4.5h	5h	mean
1	0.41	0.63	0.52	1.63	-	1.63
2	0.53	0.51	0.52	3.57	3.65	3.60
3	0.50	0.46	0.48	1.72	1.68	1.70
4	0.43	0.43	0.43	0.72	0.78	0.75
5	0.40	0.45	0.43	1.24	1.28	1.26
Mean	0.45	0.50	0.48	1.78	1.85	1.79
SD	0.06	0.08	0.05	1.08	1.26	1.08

The [D_3]glutamate flux during IV infusion on d 2 was 1900 ± 1061 μmol/(kg·h). The Q_{IV} on study d 1 did not significantly differ from Q_{IV} on d 2 (p = 0.34). Based on the rate of appearance during IV administration of [U-^{13}C]glutamate, the mean whole-body oxidation of glutamate was 1052 ± 721 μmol/(kg·h). The fractional first-pass uptake is the fraction of dietary glutamate, directly absorbed through the splanchnic system. It amounted to 77 ± 18% and 70 ± 7% on the first and second study days, respectively.

Table 3. Whole-body and splanchnic glutamate kinetics in preterm infants (n=5).

Patient	I	Q_{IV}		Q_{IG}		FFP		AFP		Mean	
		d 1	d 2	d 1	d 2	d 1	d 2	d 1	d 2	FFP	AFP
1	185	2736	1645	5084	5001	0.46	0.67	86	123	0.57	104
2	188	584	661	5003	1684	0.88	0.61	185	101	0.75	143
3	181	720	1607	5700	5298	0.87	0.70	159	125	0.79	142
4	186	1357	3575	6320	13164	0.79	0.73	146	136	0.76	141
5	173	1036	2012	6132	10705	0.83	0.81	146	138	0.82	142
Mean	183	1287	1900	5648	7170	0.77	0.70	144	125	0.74	1344
SD	6	864	1061	596	4656	0.18	0.07	36	15	0.13	28

I, glutamate intake (μmol/(kg·h)) and was equal on d 1 and 2; Q, flux (μmol/(kg·h)); FFP, fractional first pass uptake (%); AFP, absolute first pass uptake (μmol/(kg·h)); d 1, study d 1; d 2, study d 2; mean FFP and AFP, average of d 1 and 2.

Whole-body glutamate oxidation accounted for 82 ± 7% of the glutamate turnover, and the fraction of dietary glutamate that was oxidized by the splanchnic bed in first-pass was 86 ± 7%. This latter finding emphasizes that glutamate is an important source of oxidation for the splanchnic system.

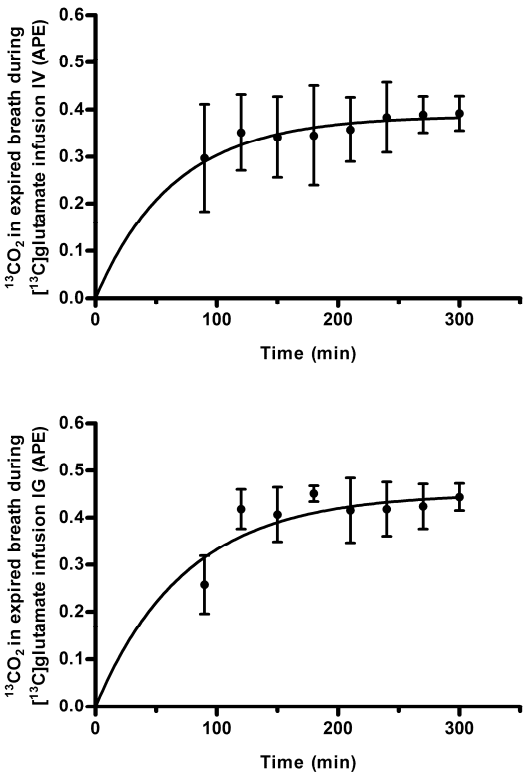

Figure 2. Enrichments of ^{13}C in expired breath during IV infusion of [U-^{13}C]glutamate on d 1 (upper) and intragastric [U-^{13}C]glutamate infusion (lower) on d 2. Rates are expressed as mean APE ± SD (n=5).

DISCUSSION

The aim of our study was to quantify the need for glutamate in the splanchnic tissues of premature infants by measuring splanchnic first-pass uptake and oxidation during full enteral feeding. Splanchnic first-pass glutamate uptake and oxidation rates were found to be very high. Approximately three fourths of the dietary glutamate intake was consumed in first-pass. Most of the extracted glutamate was then oxidized, indicating that oxidation is the metabolic fate of dietary glutamate in the premature infant.

Our results agree with those from animals (9) and human studies (11, 12). Additionally, and in agreement with our results, in this issue Haÿs et al. demonstrate that the primary fate of dietary glutamate in premature infants is splanchnic extraction (19). This finding, which

indicates that dietary glutamate is not a significant gluconeogenic substrate, is not surprising, seeing that we showed that oxidation is the primary metabolic pathway of enterally absorbed glutamate. The present data demonstrate a wide range in the mean glutamate flux, which expectedly was higher than observed in human adults. Both the IV and intragastric flux on the second study day were higher than on d 1, despite the fact that the infant's glutamate intake showed little variation. Clearly, the difference is large, yet not significant due to a small number of subjects and therefore a wide variance. However, as the first-pass splanchnic uptake showed a very small range, we may assume that the data are reliable. No such differences have been reported in animal studies (9, 20, 21). An explanation of the small range in outcome in animal studies could be the identical experimental setting or genetic similarity because animals used in these studies usually are descended from the same litter. Identical experimental setting is hard, if not impossible, to organize in human studies.

When glutamate is delivered enterally, it must pass through the enterocytes during absorption and could be used at that stage for transamination to the formation of α-ketoglutarate. The labeled α-ketoglutarate enters the Krebs cycle where the first ^{13}C label is lost by decarboxylation, thereby producing $^{13}CO_2$. However, it does not necessarily lead to complete glutamate oxidation; the remaining carbon atoms could also be used for synthesis of other metabolic compounds, eg, pyruvate, alanine and lactate. Animal studies reported that only a minor proportion of labeled pyruvate, derived from intragastric administered [U-^{13}C]glutamate, was converted to alanine and lactate and released into venous blood (1, 20). Apart from oxidation, glutamate can also act as a precursor for proline, arginine and GSH. In rats, approximately 64% of the glutamate was released as CO_2 and 10% was incorporated in amino acids such as proline, ornithine and alanine; the latter accounted only for 3% of the carbon label incorporation (20). We were not able to determine the quantity of labeled alanine, lactate and pyruvate. Thus, the contribution of $^{13}CO_2$ from oxidation from these products could not be quantified. We found a fractional glutamate oxidation of 86%, which indicates that complete oxidation is unlikely. However, from animal data, we hypothesize that the contribution of labeled CO_2 from these amino acids is relatively small and that the fractional glutamate oxidation is not overestimated. Reeds et al. (9) showed in infant piglets infused with [U-^{13}C]glutamate [M+5] that lower mass isotopomers appeared in plasma and determined the degree to which the ^{13}C label is recycled in intermediary metabolism: 11% of the labeled glutamate appeared in [M+1] and [M+3] isotopomers. We, however, were not able to determine the present abundance of plasma [$^{13}C_3$]glutamate [M+3] because the [D_3]glutamate tracer has a mass of [M+3] as well and thus could not be distinguished from the [$^{13}C_3$]glutamate produced from intermediary metabolism. Nevertheless, plasma [D_3]glutamate enrichment on d 1 is very low (0.48 MPE) and contribution of 3-carbon products (M+3) from [U-^{13}C]glutamate was minimal. The same held true for the second study day.

Several animal studies have shown that enterally derived glutamate is preferentially metabolized above systemically derived glutamate (9, 20). This could have important consequences for preterm infants receiving full parenteral feeding, with nutrients bypassing

the intestine. In these infants, only systemically derived glutamate is available and uptake by the enterocytes will be diminished, resulting in a decrease in glutamate as an energy substrate. Conversely, they have lower energy requirements (90 kcal/(kg·d)) than preterm infants who receive full enteral feeding (120 kcal/(kg·d)) (22). This difference may be due to the energy needs of the splanchnic tissues, loss of nutrients, or intestinal (glyco-)protein synthesis and thus energy storage during enteral nutrition. In general, approximately 8-14 kcal/(kg·d)) is lost in stools (23). Fuel sources used by the gut to generate energy for synthesis purposes could be glucose, amino acids, or fatty acids. We showed earlier that glucose is an important intestinal energy source for preterm infants (18). The splanchnic tissues use one third of the dietary glucose in first-pass and approximately 80% of this amount is oxidized; thus, most of the glucose is used for energy generation. The same pattern is found for glutamate, with 86% of the dietary glutamate oxidized in first-pass, approximately 14 mmol glucose/(kg·d) is oxidized in the splanchnic tissues, accounting for 10 kcal/(kg·d). Glutamate first-pass oxidation yields an additional 1.7 kcal/(kg·d). Thus, besides glucose, glutamate oxidation by the splanchnic tissues is an important source of energy. It seems likely that the glutamate demand depends on other available oxidative substrates as well and that the splanchnic glutamate oxidation rate is related to the dietary glutamate load. In piglets, we found that the intestinal glutamate oxidation was significant lower during low protein feeding compared with normal protein intake (10). We, therefore, expect that the splanchnic fraction of glutamate oxidation will increase if more dietary glutamate is provided.

Although glutamate is a nonessential amino acid, its availability has many clinical implications. Apart from oxidation and incorporation into proteins, glutamate is a precursor of the synthesis of several other amino acids and for GSH synthesis. This is a tripeptide consisting of glutamate-cysteine-glycine and is the most important intracellular antioxidant; especially in preterm infants receiving supplemental oxygen causing increased oxidative stress. Mucosal and luminal GSH both play an evident role against oxidative damage in the mucosa resulting from peroxidative damage and dietary toxins (24). Reeds et al. (8) showed that enterally derived glutamate is the preferential source for mucosal GSH synthesis in neonatal piglets. Approximately 85% of the mucosal GSH-glutamate derived from dietary glutamate, and at 500-600% per day, the fractional GSH synthesis rate was extremely high. If the intestine oxidizes almost all dietary glutamate, the quantity of glutamate left may be insufficient to maintain the preterm infant's demand for GSH production. About 135 µmol glutamate/(kg·h) is used by the enterocytes in first-pass and 116 µmol glutamate/(kg·h) thereof is oxidized. Thus, a maximum of 19 µmol glutamate/(kg·h) is left for intestinal GSH synthesis or for other proteins. In neonatal piglets GSH synthesis required the provision of 25 µmol glutamate/(kg·h) with a four times higher intake. The minimal glutamate requirement for GSH synthesis during stress and illness in human neonates is not yet known. Animal data, however, strongly indicate that mucosal GSH-glutamate derives mainly from dietary glutamate. This could have implications for parenterally fed infants as they lack supply of luminal (dietary) glutamate during parenteral feeding. The intestinal GSH synthesis may be restricted and therefore limit protection against oxidative injury.

Another important question is whether glutamate should be added to oral rehydration solutions (ORS). The standard ORS solutions (World Health Organization recommended) nowadays contain glucose and electrolytes but not glutamate. However, if the gut is at risk during diarrhea, glutamate might play an important role in restoring essential functions of the enterocytes as energy source and a precursor of protein synthesis and possibly also of increased GSH production. However, glutamate added to ORS will increase osmolarity. Clinical studies are needed to determine the precise effects of added specific amino acids such as glutamate.

A limitation of this study is that we determined glutamate kinetics in only five premature infants. However, the results demonstrate clearly that the splanchnic bed sequesters most of the enteral glutamate tracer in first-pass. We hypothesized that the intestine has a very high first-pass effect and is a major site of oxidation in neonates. The results of this study demonstrate that the splanchnic tissues are indeed a major site of metabolism and oxidation.

APPENDIX

Glutamate flux during IV infusion was calculated as follows (7):

$$Q_{IV} = i_{glu\_iv} \times [(IE_i/IE_p)-1] \quad (1)$$

where Q_{IV} is the flux of the IV infused tracer (μmol/(kg·h), i_{glu_iv} is the IV glutamate infusion rate (μmol/(kg·h) and IE_i and IE_p are the isotopic enrichments (mol% excess) of IV administered [U-^{13}C or D_3]glutamate in the infusate or in plasma at steady state, respectively.

The flux for the intragastrically administered tracer was calculated as follows:

$$Q_{IG} = i_{glu\_ig} \times [(IE_i/IE_p)-1] \quad (2)$$

where Q_{IG} is the flux of the intragastric infused tracer (μmol/(kg·h), i_{glu_ig} is the intragastric glutamate infusion rate (μmol/(kg·h) and IE_i and IE_p are the isotopic enrichments (mol% excess) of intragastrically administered [U-^{13}C or D_3]glutamate in the infusate or in plasma at steady state, respectively.

First-pass glutamate uptake was calculated as:

$$U = [(Q_{IG} - Q_{IV})/Q_{IG}] \times I \quad (3)$$

where U is the first-pass glutamate uptake (μmol/(kg·h), Q_{IG} is the flux of the intragastrically administered glutamate tracer (μmol/(kg·h), Q_{IV} is the flux of the IV administered glutamate tracer (μmol/(kg·h) and I is the intake of enteral glutamate (μmol/(kg·h).

Whole-body CO_2 production was estimated as:

$$CO_2 \text{ production} = I_B \times [(IE_{iB}/IE_B)-1] \quad (4)$$

where I_B is the infusion rate of [^{13}C]sodium bicarbonate (μmol/(kg·h), IE_{iB} is the enrichment (mol% excess) of [^{13}C]bicarbonate in the bicarbonate infusate and IE_B is the $^{13}CO_2$ enrichment at steady state during the labeled bicarbonate infusion (mol% excess). Splanchnic and whole-body glutamate oxidation rates were determined under the assumption that CO_2 production during [^{13}C]bicarbonate infusion equals the labeled glutamate tracers infusion. Assuming a constant CO_2 production rate during the 7 h of infusion, it is not necessary to use a correction factor for retention of [^{13}C]bicarbonate in the body (25).

Glutamate oxidation was calculated by multiplying recovery of [^{13}C]glutamate in expiratory air with the rate of appearance of glutamate (7, 25). The fraction of glutamate oxidized using the enrichment of the IV [U-^{13}C]glutamate infusion on study d 1 and intragastric [U-^{13}C]-glutamate on study d 2 was calculated as:

$$\text{Fractional glutamate oxidation} = [IE_{glu} \times i_B]/[IE_B \times i_{glu} \times 5] \quad (5)$$

where IE_{glu} and IE_B are $^{13}CO_2$ enrichments (mol% excess) in expired breath at steady state during IV (d 1) or intragastric (d 2) [U-^{13}C]glutamate and [^{13}C]bicarbonate infusion. The i_{glu} is multiplied by a factor 5 to account for the number of labeled C atoms.

Whole-body glutamate oxidation (d 1) was calculated as:

$$\text{Whole body glutamate oxidation} = \text{eqn } (5)_{IV} \times \text{eqn } (1)_{IV} \quad (6)$$

Acknowledgments

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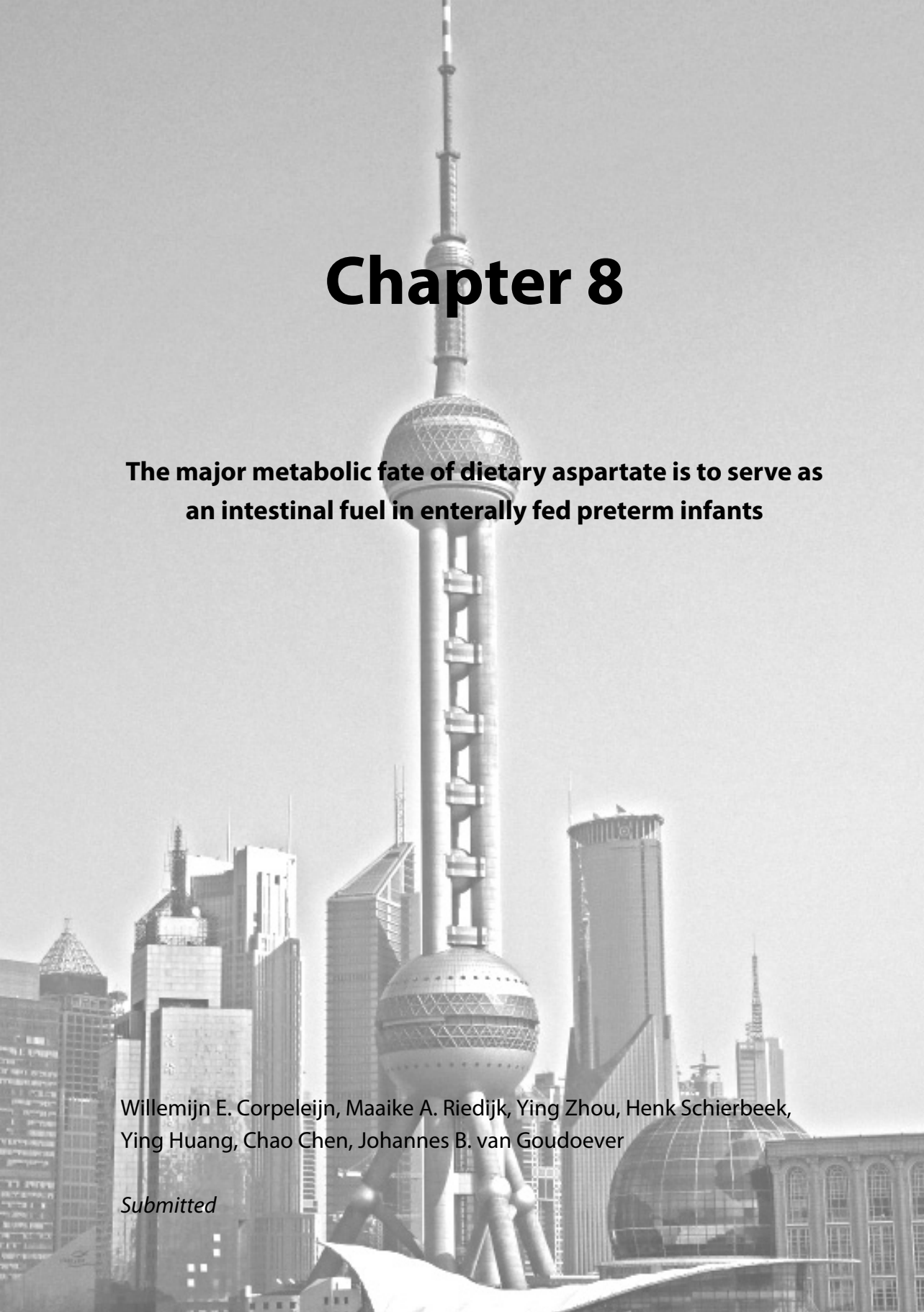

Chapter 8

The major metabolic fate of dietary aspartate is to serve as an intestinal fuel in enterally fed preterm infants

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Submitted

ABSTRACT

The intestine is a major site of amino acid metabolism, especially in neonates. Neonatal animals derive the energy needed for metabolic processes from dietary glucose and amino acids. Rats were found to oxidize aspartate in the intestine as well at a high rate. We have previously found that glutamate and glucose are important sources of energy for the splanchnic tissues in preterm infants receiving full enteral feeding. However, no data are available on splanchnic aspartate metabolism in human preterm infants.

The aim of our study was to investigate whole-body and splanchnic aspartate metabolism and to determine the primary splanchnic metabolic fate of aspartate.

Eight, enterally fed, preterm infants (birth weight 1.34 kg ± 0.43 SD, gestational age 31 wk ± 3 SD, range: 26-34 wk) were studied by dual tracer ([U-^{13}C]aspartate and [D_3]aspartate) techniques. Splanchnic and whole-body aspartate kinetics were assessed by plasma isotopic enrichment of [U-^{13}C] and [D_3]aspartate and breath $^{13}CO_2$ enrichments.

Splanchnic first-pass aspartate uptake was almost complete (77 ± 15%). Almost all (80 ± 9%) of the aspartate used in first-pass is directed toward oxidation.

In conclusion, the splanchnic tissues extract almost all of the dietary aspartate in preterm infants. As is the case for glutamate, in preterm infants receiving full enteral feeding the major fate of dietary aspartate is oxidation within the splanchnic tissues in first-pass metabolism.

INTRODUCTION

In utero the fetus obtains nutrients from cord blood. After birth the term newborn receives nutrients from breastfeeding or formula feeding, resulting in a shift from glucose as the main source of energy to a mixture of carbohydrates and fat. During enteral nutrition the gut is essential in digesting and absorbing nutrients. Consequently, maturation of the gastrointestinal tract is of crucial importance in the adaptation from intrauterine to extra uterine life. As preterm infants still have an immature gut, however, most of them will receive total parenteral nutrition (TPN) during the first period of life. Although in theory mimicking fetal nutrition, TPN is associated with high morbidity, e.g. cholestasis (1) or sepsis (2). It is, therefore, advisable to switch to enteral route of feeding as soon as possible. Transition from parenteral to enteral substrates has large implications for the energy balance. Energy expenditure and the use of substrates by the portal-drained viscera (spleen, stomach, intestines and pancreas; PDV) are determined by the route of nutrient administration. For example, enteral nutrition stimulates the secretion of gut hormones (3) and causes intestinal protein accretion and intestinal growth in neonatal pigs (4). The high impact of these effects becomes clear from the fact that the PDV account for less than 5% of the bodyweight but are responsible for up to 35% of whole body energy expenditure during enteral feeding (5). In the late seventies and early eighties, Windmueller and Spaeth found that dietary glutamate, aspartate and glucose and arterial glutamine are the major intestinal fuel sources in rats (6-8). In addition, we found that in neonatal piglets the PDV extract almost all (>90%) of the glutamate, glutamine and aspartate in first-pass (9). Since these amino acids do not appear in the portal vein we hypothesize that they are utilized by the PDV for oxidative purposes and energy generation. Research efforts have been directed at elucidating the substrate use of the human neonatal intestine. Van der Schoor et al. (10) found that in preterm infants approximately one-third of dietary glucose is utilized in first-pass during full enteral feeding and more than three-quarters is directed toward oxidation. Recently, we documented a similar fate for dietary glutamate: three-quarters was taken up by the intestine in first-pass metabolism and almost all of that was channeled toward oxidation (11). In contrast, the indispensable amino acid threonine was almost completely utilized in first-pass metabolism but was not used for oxidation (12). Clearly, these findings indicate that dispensable amino acids are important substrates for energy supply in the intestine.

Aspartate is a dispensable amino acid, which means that it can be synthesized de novo. It serves as a major nitrogen transporter by transamination and is the second amino group donor in the urea cycle. Furthermore, aspartate is an excitatory neurotransmitter and is involved in gluconeogenesis.

In general purpose of the present study was to quantify splanchnic and whole-body aspartate metabolism in premature infants on full enteral feeding. Having this knowledge will enable to meet the specific nutritional requirements of the premature neonate and to develop strategies to optimize gut function early after birth. We hypothesized that like glutamate, aspartate is also a major oxidative fuel for the intestine.

METHODS

Subjects

Eligible patients were premature infants with a birth weight ranging from 1000 to 2000 grams born in the Children's Hospital, Fudan University, Shanghai or the Erasmus MC - Sophia Children's Hospital Rotterdam or having been admitted to the NICU of either hospital < 24 hours after birth. Exclusion criteria were congenital metabolic disease, congenital intestinal disease, malformations and abnormal liver or kidney function. The study protocol was approved by the Fudan Hospital Ethical Review Board (Shanghai, PR China), the Erasmus MC Institutional Review Board (Rotterdam, the Netherlands) and the Dutch Central Committee on Research Involving Human Subjects (the Hague, the Netherlands). The study was conducted according to European Good Clinical Practice Regulations, the Declaration of Helsinki and relevant Ethical and Regulatory considerations. All infants were clinically stable at the time of the study and tolerated full enteral feeding. Feeding regimen was according to standard clinical practice at the participating hospitals. All infants received a standard preterm formula (Nenatal Start, Nutricia, Zoetermeer, the Netherlands, 75 kcal/100 mL) as the sole nutrition starting at least 24 h before start of the study until completion.

Protocol

To investigate splanchnic and whole body aspartate kinetics, a crossover study design was performed using dual tracer techniques on two separate days. Between the study days one intervening day was planned to allow the isotopes to be washed out. The general outline of the study is depicted in Figure 1. During both study days infants were implanted with an intravenous catheter for stable isotopes infusion. Starting one hour before the onset of the tracer protocol, infants were continuously fed through an intragastric tube until completion. All received the same feeding regimen on both study days.

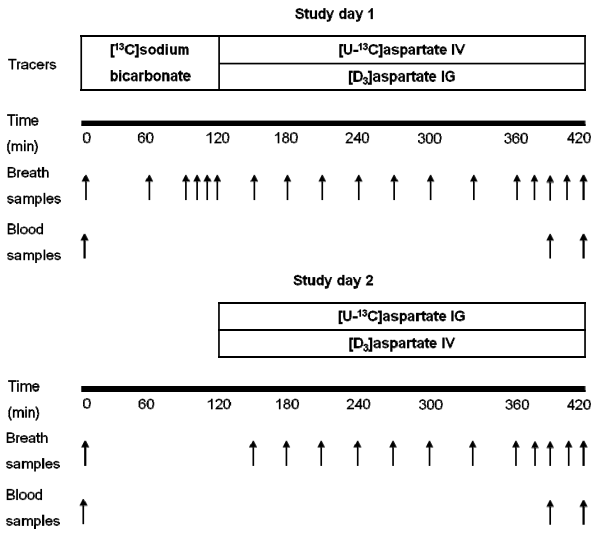

Figure 1. Study design.

Breath sampling was performed by the direct sampling method as described previously (13, 14). In brief, a 6F gastric tube (6 Ch Argyle; Cherwood Medical, Tullamore, Ireland) was placed 1 to 1.5 cm into the nasopharynx and end-tidal breath was taken slowly with a syringe connected to the end. Collected air was transferred into 10 mL sterile, non-silicon-coated evacuated glass tubes (Van Loenen Instruments, Zaandam, the Netherlands) and stored at room temperature until analysis.

During the first study day three different stable-isotopes infusions were performed. First, a primed (10 µmol/kg) continuous (10 µmol/(kg·h) intravenous infusion of [^{13}C]bicarbonate (99.0 mol% ^{13}C; Cambridge Isotopes, Woburn, Massachusetts, USA) dissolved in sterile saline and was administered intravenously for two hours. This served to assess individual whole-body CO_2 production. The ^{13}C labeled bicarbonate infusion was immediately followed by two primed 5-h infusions (15 µmol/kg and 15 µmol/(kg·h)) of [U-^{13}C]aspartate (98.0 mol% ^{13}C; Cambridge Isotopes) intravenously and (15 µmol/kg and 15 µmol/(kg·h)) of [2,2,4-D_3]-aspartate (98.0 mol% D_3; Cambridge Isotopes) enterally. The route of labeled aspartate administration was switched on study day 2. The simultaneous infusion of different labeled aspartate allowed us to determine whole-body and splanchnic aspartate kinetics. All tracers were found to be sterile and pyrogen free before use in our studies. Baseline blood and breath samples were collected at time 0. Breath samples were collected every 30 minutes. During the last hour of each tracer infusion, breath samples were collected at 15-min intervals and two blood samples were obtained after 4.5 h and 5 h tracer infusion. Blood samples were collected by arterial blood puncture and blood was centrifuged immediately. Plasma samples were stored at -80ºC until further analysis. The total amount of blood withdrawn per study day was 1.5 mL, which is less than 2% of total blood volume in a 1000-g infant.

Analytical Methods

Plasma enrichments of [U-$^{13}C_4$]aspartate and [2,2,3-D_3]aspartate were measured by gas chromatography-mass spectrometry (GC-MS), using *N*-methtyl-*N*-(tert-butyldimethyl)silyl derivates. Briefly, 100 µL of plasma was deproteinized with 100 µL of 0.24 M sulfosalicyclic acid. After centrifugation (for 8 min at 4°C, and 14,000x *g*), the supernatant was passed over a Dowex cation-exchange resin column (AG 50W-X8, hydrogen form, Bio-Rad Laboratories, Richmond, CA). The column was washed with 3 mL of water and the amino acids eluted with 1.5 mL M NH_4OH. The eluate was dried at room temperature in a speedvac (Savant, Thermofisher, Breda, the Netherlands) and derivatives of the amino acids were finally formed by adding 25 µL aceto-nitrile and 50 µL of MTBSTFA (Pierce, Omnilabo, Breda, the Netherlands) and incubation at 60°C for 60 min. Analyses were performed on a Trace gas chromotograph coupled with a Thermo DSQ mass spectrometer (Interscience BV, Breda, the Netherlands) by injecting 0.5 µl on a PTV injector and a 30 m x 0.25-mm VF-17ms 0.25 µm coated fused silica capillary column (Varian, Middelburg, the Netherlands). Ion abundance was monitored by selective ion monitoring, and mass to charge (m/z) for natural aspartate [M+0] at m/z 418.3 and for [2,3,3-D_3]aspartate [M+3] and [U-$^{13}C_4$]aspartate [M+4] at m/z 421.3 and 422.3, respectively.

$^{13}CO_2$ isotopic enrichment in expired air was measured by isotope ratio mass spectrometry

(ABCA; Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands) and expressed as atom percent excess (APE) above baseline. APE was plotted relative to time. Steady state was defined as three or more consecutive points with a slope not different from zero.
$^{13}CO_2$ isotopic enrichment in expired air was measured by isotope ratio mass spectrometry (ABCA; Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands) and expressed as atom percent excess (APE) above baseline. APE was plotted relative to time. Steady state was defined as three or more consecutive points with a slope not different from zero.

Calculations

We studied amino acid turnover according to Waterlow's stable isotopic dilution model (15). Whole-body CO_2 kinetics were calculated as described previously (16). Substrate turnover was calculated by using dual stable isotope tracer techniques. Tracer dilutions were measured during steady state as described previously (10-12, 17, 18). The equations used to obtain the results are detailed in the Appendix.

Statistics

Data are expressed as mean ± SD values or as medians and 25th and 75th percentiles obtained from samples taken over the last hour of each tracer infusion. Differences in background and steady-state enrichments between the two study days were analyzed by paired *t* tests. A value of $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed using SPSS software (version 14.0; SPSS Inc, Chicago, IL).

RESULTS

Eight preterm infants were included. Their characteristics are listed in Table 1. Mean gestational age was 31 ± 3 wk (range: 26 - 34 wk) and mean birth weight was 1.34 ± 0.43 kg. Patients 1-6 were included in the Fudan Children's Hospital and patients 7-8 in the Erasmus MC-Sophia Children's Hospital. All were clinically stable at the time of both study days and were breathing spontaneously. They all tolerated enteral feeding well and none of them had sepsis or unstable temperature, as these were exclusion criteria. Average aspartate intake was 109 ± 5 μmol/(kg·h).
Mean baseline $^{13}CO_2$, expressed as atom percent excess (APE), on study d 1 (1.0871 ± 0.0016 APE) did not significantly differ ($p = 0.2$) from that on d 2 (1.0878 ± 0.0012 APE). Baseline plasma enrichments of [U-^{13}C]aspartate and [2,2,4-D_3]glutamate did not significantly differ between the two study days ($p = 0.08$, $p = 0.5$, respectively).
Splanchnic and whole-body kinetics were calculated from plasma aspartate enrichments (Table 2) and $^{13}CO_2$ enrichments in expired breath during steady-state. As shown in Figure 2, the $^{13}CO_2$ excretion in breath reached an isotopic plateau during both IV and IG [U-^{13}C]-aspartate infusions on d 1 and d 2, respectively. During IV infusion the slope of the line from time point 300 min to 420 min was not different from zero ($p = 0.5$). Also during IG infusion the slope of the line was not significantly different from zero ($p = 0.9$). Although we obtained

only two blood samples during the tracer infusions, we assumed that plasma enrichment also had reached steady-state because the $^{13}CO_2$ enrichment in breath was at isotopic plateau.

Table 1. Patient characteristics.

Patient	GA wk	BW kg	SA 1 d	SA 2 d	SW 1 kg	SW 2 kg	Intake ml/(kg·d)	Energy Kcal/(kg·d)
1	34	1.50	28	30	1.79	1.79	161	121
2	33	1.24	39	41	1.63	1.63	162	122
3	33	1.82	17	19	1.90	1.98	158	119
4	30	1.53	25	27	1.69	1.69	156	117
5	32	1.30	19	21	1.31	1.44	165	124
6	33	1.87	7	9	1.84	1.84	157	118
7	29	0.82	28	30	1.09	1.13	142	107
8	26	0.67	27	29	0.79	0.86	146	110
Mean	31	1.34	24	26	1.50	1.54	110	117
SD	3	0.43	9	9	0.40	0.38	6	6

GA, gestational age; BW, birth weight; SA 1, postnatal age at day study day 1; SA 2, postnatal age at study day 2; SW 1, weight at study day 1; SW 2, weight at study day 2.

Table 2. Steady-state isotopic enrichments of [U^{13}C]aspartate and [D_3]aspartate in plasma on study d 1 and 2.

	D1	D2
[U-^{13}C]apartate (mol% excess)	9.6 (7.5-12.4)	2.6 (1.9-3.0)
[D_3]aspartate (mol% excess)	1.2 (0.9-1.8)	7.8 (4.7-8.8)
Q_{IV} (µmol/(kg·d))	129 (100-170)	156 (131-502)
Q_{IG} (µmol/(kg·d))	1181 (832-1851)	419 (332-485)

Rates are expressed as median; 25th–75th percentile in parentheses.

Table 3 shows the results of whole-body and splanchnic kinetics. The mean estimated whole-body CO_2 production was 28.97 ± 1.54 mmol/(kg·h). The mean rate of appearance, or flux (Q) of [U-^{13}C]aspartate, administered intravenously was 129 (100-170) µmol/(kg·h) on d 1 and 156 (131-502) µmol/(kg·h) on d 2. The splanchnic fractional first-pass uptake of aspartate is the fraction of aspartate directly taken up from the diet by the splanchnic tissues (intestine and liver) and which therefore does not appear in the systemic circulation. It amounted to 90 ± 3% on d 1 and 64 ± 10% on d 2 (mean d 1 and d 2: 77 ± 15%). Whole-body aspartate oxidation was calculated from the flux of the intravenously infused [U-^{13}C]aspartate (equation 6) on day 1. The median whole-body oxidation was 80 ± 9 µmol/(kg·h). The fraction of aspartate oxidized by the splanchnic tissues in first-pass was 81 ± 8% on the first study day and 80 ± 11% on the second day (mean d 1 and d 2: 80 ± 9%). This result confirms our hypothesis that aspartate, like glutamate, is an important fuel for the intestine of premature neonates.

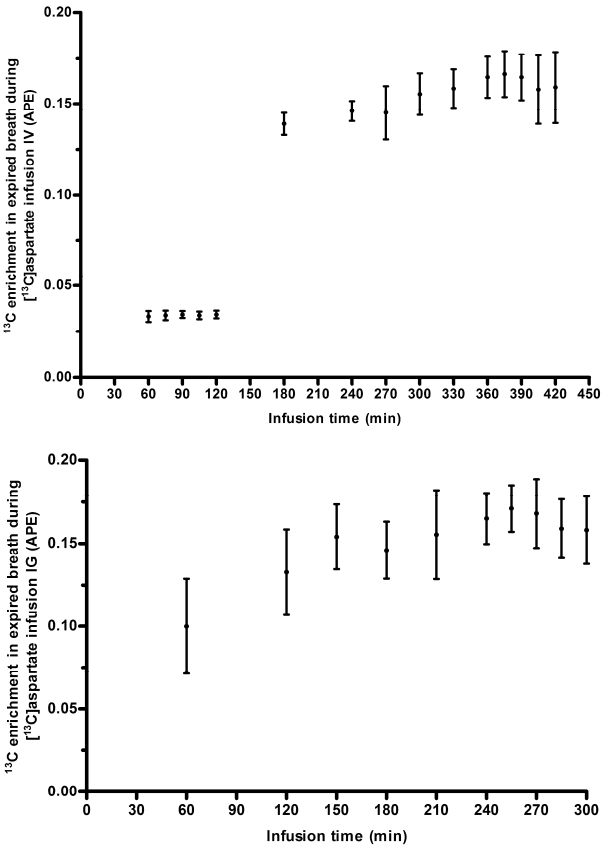

Figure 2. Enrichments of expired air during labeled aspartate infusion. Enrichments of ^{13}C in expired breath during IV [U-^{13}C]aspartate infusion on d 1 (upper) and IG [U-^{13}C]aspartate infusion on d 2 (lower). Rates are expressed as mean atom percent excess APE ± SD (n=8).

Table 3. Whole-body and splanchnic aspartate kinetics in preterm infants.

Patient	I	FFP		FFP	WB oxidation
		d 1	d 2	Mean	
1	113	87	68	78	55
2	114	86	74	80	46
3	111	94	54	74	139
4	110	89	69	79	92
5	111	88	60	74	87
6	110	89	75	82	100
7	100	88	47	68	168
8	103	95	62	79	408
Mean	109	90	64	77	80
SD	5	3	10	15	9

I, aspartate intake (μmol/(kg·d)) and was equal on d 1 and 2; FFP, fractional first-pass aspartate uptake (%); WB oxidation, whole-body oxidation during IV infusion on d 1 (μmol/(kg·d)); d 1, study day 1; d 2, study day 2; mean, average of d 1 and 2.

DISCUSSION

The present study describes the splanchnic aspartate kinetics in preterm infants receiving full enteral feeding. Almost all dietary aspartate was taken up by the splanchnic tissues in first-pass metabolism and there was a high fractional oxidation of the sequestered aspartate. Our results are in concordance with those of earlier studies performed in animals (7-9). The gut is an important modulator of whole-body amino acid fluxes. It controls amino acid absorption and is thought to modulate the metabolic fates of absorbed amino acids. During enteral feeding gastrointestinal tissues are the first to be exposed to nutrients so this tissues' needs are met first. The possible metabolic fates of the sequestered amino acids in the splanchnic tissues in first-pass are: protein synthesis, intermediate metabolism, and irreversible oxidation to CO_2 and ammonia. Amino acids incorporated into new (glyco-) proteins and secreted in the gut lumen could become systemically available after recycling (19). Amino acid requirement of neonatal piglets receiving enteral feeding is significantly higher (25-50%) than of parentally fed neonatal piglets (20-22). In enterally fed piglets it has been shown that approximately 50% of dietary amino acids appears in the systemic circulation after first-pass, but with substantially differing proportions for the between individual amino acids (9).

All these findings stress the need to supply the (premature) neonate's gut with adequate amounts of "fuel", like aspartate, glutamate and glucose. This might prevent the splanchnic tissues from oxidizing essential amino acids like lysine (16), which otherwise would result in depletion of essential amino acids and consequently in inhibition of protein synthesis. Our results show that the intestine sequesters most of the enteral aspartate. Thus, most of the systemic aspartate available must derive from de novo synthesis in the body. The quantitatively most important aspartate synthesis pathway remains unknown. It could involve, for example, transamination of oxaloacetate or the removal of an amide group of asparagine. After premature birth most infants are fed with TPN during the first days of life, and consequently almost all nutrients are surpassing enteral metabolism. During TPN, aspartate is administered intravenously as a constituent of parenteral nutrition solutions. The metabolic fate of parenterally administered aspartate needs to be investigated in future studies.

Apart from whole-body aspartate oxidation, we were also able to determine splanchnic aspartate oxidation. Our findings confirm the results from animal studies, which showed intestinal aspartate oxidation ranging from 39 to 52% (7, 8). As early as the 1970s Windmueller and Spaeth administered radio-actively labeled aspartate (L-[U-^{14}C]aspartate) to isolated segments of rat jejunum and measured the release of ^{14}C labeled products into the blood (7). In concordance with our results the most important ^{14}C labeled product released was $^{14}CO_2$. Next came labeled lactate, glucose and alanine. Intragastric administration of [U-^{13}C]aspartate theoretically allows to determine aspartate metabolites, so that the abovementioned substrates can be quantified. Because of the tremendous dilution rate, high amounts of labeled aspartate would be needed to measure the metabolites of quantitatively minor importance. We were not able to determine labeled alanine during simultaneous administration of [2,3,3-D_3]aspartate because this tracer has a mass of [M+3] as well and

therefore, cannot be distinguished from the formed labeled [$^{13}C_3$]alanine in intermediary metabolism.

Aspartate has a very high turnover rate and it is known that the pool of dispensable amino acids is large due to endogenous synthesis. Aspartate has many functions in the metabolism of the cell. Aspartate enters the urea cycle as the second amino group donor in the liver and condensates with citrulline to form arginosuccinate. Arginosuccinate is the precursor of arginine, a possible conditionally essential amino acid during periods of rapid cell growth (e.g. after premature birth). Depending on the cells energy status, aspartate either enters the Krebs cycle by prior metabolism to oxaloacetate, or it forms alanine which can be used to form pyruvate and subsequently glucose. It is not known if alanine is formed by transamination of pyruvate or if there is release of CO_2 under the influence of the cytosolic enzyme aspartate 4-decarboxylase. Stoll et al. (23) showed very low net portal balance of aspartate (4-24% of the intake) in neonatal piglets. That of alanine and that of arginine, however, was >100%. This suggests that there is a net production of these amino acids by the PDV. Aspartate is also a precursor of asparagine, another non-essential amino acid. It is formed by transfer of an amide group from glutamine to aspartate, which reaction requires ATP. Aspartate also plays a crucial role in purine and pyramidine synthesis as this is dependent on the donation of its amino group.

A limitation of this study is the small number of infants studied: only eight. Nevertheless, we do believe that these data are valid, as both the fractional first pass up-take and the fractional oxidation rate do not demonstrate a wide range. In conclusion, the results of this study demonstrate that there is a high uptake of dietary aspartate by the splanchnic tissues in first-pass, most of which is used for oxidative purposes.

APPENDIX

We used similar calculations for aspartate metabolism as we did for glutamate, lysine and glucose kinetics (10, 11, 18):

$$Q_{iv}=I_{asp\_iv} \times [(IE_{inf}/IE_p)-1] \quad (1)$$

Where Q_{iv} is the flux of the intravenously infused aspartate (µmol/(kg·h)), I is the infusion rate (µmol/(kg·h)), IE_{inf} is the enrichment of the infusate (mol% excess) and IE_p is the enrichment found in plasma (mol% excess).

Aspartate flux of intragastrically infused aspartate was calculated as:

$$Q_{ig}= I_{asp\_ig} \times [(IE_{inf}/IE_p)-1] \quad (2)$$

Where Q_{ig} is the flux of the intragastrically infused aspartate (µmol/(kg·d)), I is the infusion rate (µmol/(kg·h)), IE_{inf} and IE_p are the enrichments (mol% excess) found in plasma and of the

infusate, respectively.

First-pass aspartate uptake was calculated as:

$$U = [(Q_{ig} - Q_{iv})/Q_{ig}] \times I \quad (3)$$

Where U is the first pass uptake (μmol/(kg·h)), Q_{ig} is the flux of the intragastrically infused tracer, Q_{iv} is the flux of the intravenously infused tracer, and I is the intake of enteral aspartate intake (μmol/(kg·h)).

Whole-body CO_2 production was estimated as:

$$\text{Whole-body } CO_2 \text{ production} = I_B \times [(IE_{iB}/IE_B) - 1] \quad (4)$$

Where I_B is the infusion rate of the labeled bicarbonate μmol/(kg·h)) , IE_{iB} is the enrichment of the infused bicarbonate (mol% excess) and IE_B is the enrichment in expired CO_2 (mol% excess) .
This equation does not correct for [^{13}C]bicarbonate in different body pools and will overestimate CO_2 production rate. However, the same correction factor has to be applied to quantify the aspartate oxidation rate. Consequently aspartate oxidation rate is not overestimated.

Fraction of aspartate oxidized was calculated as:

$$\text{Fraction of aspartate oxidized} = [IE_{asp} \times i_B] / [IE_B \times i_{asp} \times 4] \quad (5)$$

Where IE_{asp} is the enrichment of CO_2 in expired breath during [U-^{13}C]aspartate infusion (on day 1 intravenously and on day 2 intragastrically infused), IE_B the enrichment of CO_2 during [^{13}C] bicarbonate infusion (on day 1), i_B is the infusion rate of [^{13}C bicarbonate and I_{asp} is the infusion rate of the [U-^{13}C] aspartate (μmol/(kg·h)).
Enrichment of aspartate is multiplied by 4 to account for the number of labeled C-atoms in [U-^{13}C]aspartate.

Whole-body aspartate oxidation is calculated as:

$$\text{Whole-body aspartate oxidation} = \text{Equation 5} \ X \ \text{Equation } 1_{IV} \quad (6)$$

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General discussion and summary

Chapter 9

General discussion

INTRODUCTION

Within the last decades much effort has been put into improving care for the preterm infant. Since the introduction of surfactant, antenatal steroids, specialized perinatal care and the improvement of mechanical ventilation the survival of preterm infants has increased substantially, even for very preterm infants (< 27 wk gestational age). Over the past years, the attention has partly been shifted to nutritional aspects to optimize treatment and long term outcome of these infants. For instance, the idea to withhold preterm infants from nutrients during the first days of life has been revised. Instead, introduction of amino acids and energy directly onwards birth is necessary to achieve a positive nitrogen balance (1-4). Protein synthesis is required to ensure growth and therefore supply of amino acids and energy is needed. The optimal composition of parenteral feeding and enteral feeding has not been established yet.

Optimal feeding has a short-term effect by increasing survival and reducing complications, such as a reduction in severe chronic lung disease upon improvement of nutritional intakes in the first few weeks of life (A. Lapillonne, personal communication). Moreover, in both preterm and term infants with acute perinatal brain injury, a high-energy and- protein diet providing 120% of the estimated average recommendation (EAR), has shown increased brain growth and overall body growth in the first 12 months compared to infants receiving 100% EAR (5).

More important, a long-term effect on later development and intelligence outcome has been shown as well (6). Thus, to support anabolism, early nutrition needs to be commenced immediately after birth. In this respect, the quantity of nutrients is of special interest. It is well known that the protein requirement of the preterm infant depends on several factors, such as gestational age, birth weight and post-conceptual age. The European Society of Gastroenterology, Hepatology and Nutrition (ESPGHAN) will advise from 2008 onwards to provide preterm infants weighing less than 1800 grams with 3.8-4.5 gram protein/(kg·d) (in press, J Pediatr Gastroenterol Nutr 2008). This is substantially higher than the former recommendation by the American Academy of Pediatrics, which was 3.5-4.0 g protein/(kg·d) (7).

Amino acid composition and protein amount of human milk varies widely and is also dependent on the time of birth (gestational age) and the post-conceptual age. Breast milk from mothers given birth prematurely contains more protein compared to milk from mothers given birth at term during the first few weeks post partum (Fig. 1). At around 2-4 weeks the composition is identical.

Thus, preterm infants fed their mother's milk need additional breast milk fortifier (BMF) or an increase in fluid intake up to 250 mL/(kg·d) to guarantee adequate daily protein intake. However, calcium, phosphate, and several vitamins are not sufficiently provided (11, 12), even at such high intakes. Sometimes, the clinical condition of the infant does not permit an intake > 140 mL/(kg·d), eg during bronchopulmonary dysplasia. The current amino acid recommendations for enterally fed preterm infants are based on the body composition of the fetus in utero (13). However, it is necessary to define these recommendations on actual

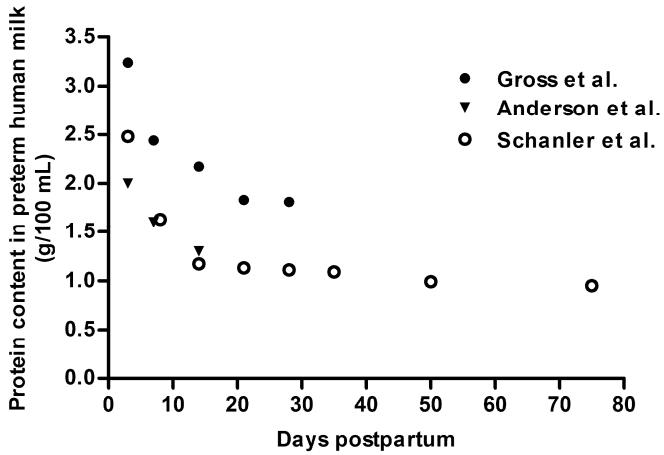


Figure 1. Protein content in preterm human milk in the first four weeks. Based on Gross et al. (8), Anderson et al. (9) and Schanler et al. (10).

requirements. The nutritional requirements of preterm infants differ from term infants because of illness, transition to extra-uterine environment and immaturity of enzymes, among other factors. In addition, by using the composition of fetal proteins as a gold standard, the requirement will give incomplete information since amino acids are not only needed for growth but are also involved in synthesis of functional glyco-proteins, like mucins, glutathione and proteins with a short lifetime such as fibrinogen and C-reactive protein. The need for specific age related nutritional requirements is increasing, since we are able to treat infants with decreasing gestational age. For instance, it is doubtful if the requirement for an infant born at gestational age of 25 wk is similar to that of a preterm infant of 33 wk since, there is a difference in the activity of several metabolic pathways such as cysteine synthesis.

In the following section we discuss the major findings presented in this thesis in relation to other studies. Finally, we discuss the future research proposals arising from the findings in our present studies.

CYSTEINE

Requirements

The biochemical immaturity of preterm infants limits several biosynthetic processes. Some amino acids are therefore conditionally indispensable for some period. Conditionally indispensable indicates that the metabolic need exceeds the endogenous synthetic capacity, caused by, for example, immaturity or critical illness. Arginine, cysteine, glycine, glutamine and tyrosine are considered to be conditionally indispensible amino acids (14-16)**.** Cysteine is believed to be conditionally indispensable in preterm infants due to limited synthesis from methionine and serine, which is caused by a decreased or even absent enzyme activity due

to immaturity (17-19).

Snyderman et al. (20-24) performed a number of studies investigating the requirements of dispensable amino acids in enterally fed term and preterm infants. She was the first to report in vivo evidence that cystine is an indispensable amino acid for the premature infant (25). Rates of nitrogen retention and weight gain were decreased in 2- to 4-month-old infants born prematurely who were enterally fed a synthetic diet without cyst(e)ine. The cyst(e)ine intakes of 44 or 66 mg/(kg·d) did not restore nitrogen retention and weight gain to control values and, subsequently, she recommended a minimal intake of 85 mg cyst(e)ine/(kg·d). However, these studies were based on nitrogen balance and weight gain rate. Over the years it has become clear that nitrogen balance usually overestimates nitrogen retention and that growth rate also depends on factors other than amino acid intake. Nitrogen losses consist not only of losses through faeces and urine but also by respiration and skin. The latter two are difficult to measure and the nitrogen method is therefore not very accurate. Moreover, 7 to 10 days adaptation is needed to establish nitrogen equilibrium (26). This adaptation time of one week is another critical aspect since present clinical practice does not accept to maintain neonates on either deficient or excess amino acid intakes for a minimum of 7 days. This might have contributed to the fact that no such requirement studies have been conducted in preterm infants since that time. We therefore established the cysteine requirements for enterally fed preterm infants (**Chapter 3 and 4**) by the indicator amino acid oxidation (IAAO) method.

In the early eighties Ball and Bayley introduced the IAAO method to determine the requirement of indispensable amino acids in piglets (27). Soon, this method was expanded to human adults (28, 29). The IAAO method is non-invasive and is based on the assumption that amino acids partition between protein synthesis and oxidation. If one indispensable amino acid is deficient this will limit overall protein synthesis. Proteolysis occurs to accomplish the release of this specific amino acids and the surplus of amino acids will be oxidized (30). This non-invasive method is therefore highly suitable to investigate amino acid requirements in preterm infants. To our knowledge we are the first research group who have applied this method in enterally fed preterm infants. Pencharz et al. (31) used this method in parenterally fed preterm infants.

We hypothesize that cysteine is a conditionally indispensable amino acid during the first period of life. We found by using the IAAO that the cysteine requirements for enterally fed low-birth weight infants at postmenstrual age of 36 weeks is less than 18 mg/(kg·d) (**Chapter 3**). Our estimated enteral cysteine requirement is at least 5 times lower than Snyderman advised (25). However, Snyderman did not provide information about the methionine intake and it is possible that their recommendation might be overestimated in view of an inadequate methionine intake. Since methionine is an indispensable amino acid, a possible inadequate intake could have limited protein synthesis resulting in an impaired rate of weight gain. On the other hand, cysteine might have a sparing effect on methionine requirements in both piglets (32) and humans (33). In this regard, low but adequate methionine intake could also have resulted in a higher cysteine requirement. In addition, Zlotkin and Anderson (19) demonstrated that activity of the enzyme cystathionase in the liver

increases rapidly after birth and reaches mature levels at about 3 months of age. This finding indicates that cystathionase activity is dependent on gestational age and that the cysteine requirement will change with postmenstrual age. We hypothesized that the requirements for preterm infants change (ie decrease) with increasing postnatal age. However, in very-low-birth weight infants born at mean gestational age of 29 wk, the cysteine requirement determined at postmenstrual age of 32 and 35 wk did not differ and was again less than 18 mg/(kg·d) (**Chapter 4**). Also, the gestational age of 29 wk versus 33 wk had no effect on the cysteine requirement (**Chapter 3 and 4**). Thus, we can conclude from our studies that cysteine does not seem to be a conditionally indispensable amino acid in enterally fed very-low-birth-weight preterm infants, and that is not dependent on postmenstrual age if older than 32 wk.

The preterm infant may be susceptible to cysteine depletion due to abrupt halt of placental supply after birth. In addition, the preterm infant does not tolerate full enteral feeding immediately after birth and therefore ,generally, the first route of feeding is parenteral during the first days of life. However, parenteral nutrition solutions lack cysteine because it is unstable in solution and oxidizes easily to cystine, which is insoluble. Many standard parenteral solutions therefore contain little cysteine or no cysteine. Others contain N-acetyl-cysteine (NAC), which improves solubility dramatically. Preterm infants, however, do not seem to be capable of de-acetylating the acetylated cysteine efficiently, resulting in high plasma levels of NAC and still low levels of cysteine (34). The short and long term side effects of high NAC levels are unknown and need to be followed. To compensate for low cysteine concentrations, parenteral solutions contain relatively high levels of methionine. Consequently, elevated methionine levels are associated with total parenteral nutrition (35). Neonates receiving a high dose of methionine show high plasma methionine and low plasma cysteine concentrations, which implies that the excess of methionine is not used for cysteine synthesis (36, 37). In addition, rabbits fed a high dose of methionine intravenously (121 mg/(kg·d)) as part of their total parenteral nutrition or in addition to standard enteral feeding showed severe liver injury and thus caution should be taken to use parenteral nutrition solutions containing high dose of methionine (38). An optimal parenteral nutrition solution, especially sulfur amino acids, for the preterm infants still needs to be defined. Recently, the total sulfur amino acid requirement for parenterally fed term infants has been determined to be 49 mg/(kg·d) (Courtney-Martin et al. in press AJCN 2008).

Following Snyderman (25), several studies have attempted to document clinically that 1) cysteine is a conditionally dispensable amino acid and 2) there is a need for an exogenous source of cysteine during infancy. Most of these studies used the plasma cysteine concentration as the primary outcome measurement and were performed in parenterally fed infants. Pohlandt (39) observed no differences in plasma cystine concentrations in parenterally fed preterm infants receiving only glucose compared to infants receiving a mixture of synthetic amino acids free from cystine. In both groups plasma cystine concentrations were low, indicating that cystine synthesis was limited. Furthermore, Viňa et

al. (40) reported low plasma cysteine concentrations in premature infants fed only a parenteral solution deficient in cystine compared to term infants. On the other hand, Zlotkin et al. (41) did not find differences in nitrogen balance for parenterally fed term and preterm infants with or without cysteine supplementation indicating that cysteine was not deficient. However, they showed slightly higher urinary 3-methylhistidine excretion, which is an index for muscle protein catabolism, suggesting either muscle protein catabolism or an increase in muscle mass in cysteine supplemented infants. An evident relationship could not be detected. Malloy et al. (42) showed that cysteine supplementation increased free cysteine plasma concentration and sulfur balance in preterm infants. However, it did not improve nitrogen retention, indicating that cysteine is not a conditionally dispensable amino acid in these infants. It needs to be emphasized that these studies were done in parenterally fed infants. No data on enterally fed preterm infants are available except for Snyderman et al. (25).

In view of the above discussed studies, plasma cysteine concentration as the primary outcome needs to be discussed. A difference in plasma cysteine concentration in parenterally fed preterm infants with or without cysteine does not necessarily mean that cysteine is indispensable under specific conditions. It just shows a difference in plasma concentration but it does not indicate if there is a shortage of cysteine or that it is due to a higher consumption. Concentration is a static analysis instead of being dynamic and it is possible that the drop in plasma cysteine concentration is, for example, a result of cysteine need for glutathione synthesis. The parenterally fed neonate is usually sicker than the enterally fed infant and subsequently might have an increased oxidative stress state which requires a higher glutathione demand.

Gut metabolism

Besides the higher requirement for some nutrients due to immaturity of metabolic pathways, optimal intestinal function and integrity requires a larger quantity of indispensable as well as dispensable amino acids. A well-functioning small intestine is indispensable in maintaining optimal digestion and absorption of dietary nutrients. One of the problems in feeding the preterm infant is immaturity of the gastrointestinal tract, which is partly age dependent. As a consequence, the preterm infant is generally parenterally fed during the first period after birth because of intolerance of enteral feeding. It is well known that during parenteral feeding the gut is largely by-passed and lack of enteral nutrient support may lead to reduced growth and functional development of the intestinal mucosa (43). In this respect, low plasma cysteine concentration could also be a direct effect of total parenteral feeding. Steging and Den Besten (44) were the first to demonstrate the in vivo involvement of the gastrointestinal tract in cysteine synthesis. Human adults were fed a cystine-free diet otherwise containing adequate amounts of methionine intravenously which was immediately followed by an intragastrical infusion. They demonstrated that plasma cysteine concentrations were significantly higher in these subjects fed the cystine-free diet enterally versus intravenously. This finding strongly suggests that the splanchnic tissues (liver and intestine) are a key site for cysteine synthesis. It is of particular importance to investigate the contribution of the

gastrointestinal tissues in cysteine production in preterm infants in view of the route of feeding. If a considerable part of endogenous cysteine synthesis occurs in the intestine, the impact of parenteral feeding is unequivocal since most standard parenteral solutions do not contain cysteine. Consequently, minimal endogenous cysteine synthesis results in a cysteine deficiency. Besides, in preterm infants the cysteine consumption is high due to oxidative stress as a result of oxygen supplementation, hypoxia or sepsis, resulting in disturbances in redox state. Redox state is regulated by glutathione (GSH) and is reduced to GSSG and transported from the cell (45). The reduction of the intracellular GSH pool stimulates de novo glutathione synthesis, which requires cysteine, glutamate and glycine. Additionally, glutathione plays a role in protecting the gastrointestinal epithelia against toxicity associated with inflammatory disease, ischemia, oxidative damage and chemotherapy (46, 47). However, free cysteine has a very small pool size compared to glutamate and glycine and is therefore believed to be the rate-limiting amino acid for glutathione synthesis (48).

Further research by Shoveller et al. (32) showed that piglets enterally fed a diet containing methionine but no cysteine had significantly higher plasma cysteine concentrations than parenterally fed piglets fed the same diet. This finding suggests again that the gut is involved in cysteine synthesis. This is indirectly confirmed by the linear correlation between dietary methionine intake and plasma cysteine concentrations in the enterally, but not in parenterally fed piglets. Despite the clear evidence that splanchnic tissues play a major role in methionine metabolism, the relative contribution of the gastrointestinal tissues is not completely clear. In **chapter 5** we, therefore, investigated the metabolic fate of dietary methionine in the gastrointestinal tract of neonatal piglets. We hypothesized that the gastrointestinal tissues have an important contribution to transmethylation, remethylation and transsulfuration. Human studies conducted with stable isotopes show that a significant proportion of methionine is transmethylated and remethylated in first-pass metabolism of splanchnic tissues (49, 50). However, they did not demonstrate an active transsulfuration pathway in these tissues. In accordance with these studies, we showed, in vivo, that the gastrointestinal tissues are a metabolically significant site of methionine transmethylation and remethylation, and confirmed the involvement of the gastrointestinal tract in methionine metabolism (**Chapter 5**). In agreement with the human studies, both trans- and remethylation were significantly higher in neonatal piglets during intraduodenal than intravenous tracer infusion, which indicates first-pass methionine metabolism. In addition, we found a high rate of intracellular transmethylation, by means of loss of the deuterated methyl moiety via S-adenosylmethionine synthesis, in both intestinal and liver tissues. This might be explained by the high turnover rate of intestinal mucosa which demands a high cell proliferation. S-Adenosylmethionine dependent methylations are essential for biosynthesis of a variety of cellular components DNA and RNA, and control cell proliferation and differentiation (51, 52).
The higher plasma cysteine concentrations in enterally fed piglets compared to parenterally fed piglets (32) could also indicate that parenterally fed piglets have a higher need for cysteine for glutathione, taurine or sulfate synthesis. However, cysteine supplementation

resulted in higher plasma cysteine concentrations in parenterally than in enterally fed piglets (32). It is possible that luminal cysteine, like glutamate (53), is preferentially used for intestinal glutathione synthesis and directly channeled to glutathione. The higher plasma cysteine concentration during parenteral administration might also be explained by an inhibitory effect of dietary cysteine on the transsulfuration rate, i.e. a reduction in cysteine synthesis. In vitro studies showed that feeding cysteine reduces cystathionine ß-synthase (CBS) activity (54, 55). The same effect of dietary cysteine on transsulfuration is seen in vivo in humans (56). Additionally, dietary cysteine also reduced transmethylation (56) and induced remethylation (57). However, a clear cysteine sparing effect on methionine requirement has not yet been established

Moreover, we showed that the gastrointestinal tissues are not only a site of intracellular homocysteine synthesis but also of net release into the circulation (**Chapter 5**). Case–control as well as prospective studies have demonstrated that the plasma total homocysteine level is a strong, graded, and independent risk factor for coronary heart disease and stroke (58, 59). Homocysteine is believed to play an important role in pregnancy complications as well (60, 61). Not much research has been performed to determine to which extent the intestinal tract contributes to homocysteine synthesis. We found that approximately a quarter of the methionine uptake was released as homocysteine by the portal tissues, which points out that the intestine is a considerable organ for homocysteine production. With regard to our results, Shoveller et al. (62) also investigated homocysteine plasma concentrations in enterally and parenterally fed piglets. They showed that plasma homocysteine concentration increased linearly with higher methionine intakes and this happened regardless of the route of feeding. Furthermore, plasma homocysteine concentration was not affected in parenterally fed piglets with or without cysteine. Thus, if the gut is bypassed, additional cysteine did not result in increased plasma homocysteine levels. This confirms our hypothesis of intestinal involvement in cysteine synthesis. In contrast, enteral feeding supplemented with cysteine resulted in lower plasma homocysteine concentrations than cysteine-free feeding. The additional cysteine might have initiated a decrease in transmethylation and an increase in remethylation, which is also seen in adult humans (56). Given that the intestine plays an important role in homocysteine metabolism, this has important implications regarding the composition of infant formulas within the scope of homocysteine plasma concentrations.

Since we have demonstrated that the intestine plays an important role in the transsulfuration pathway (**Chapter 5**), we also explored the endogenous cysteine synthesis in enterally fed preterm infants (**Chapter 6**). We found, in contrast to our expectations, that, like in parenterally fed infants, plasma cystine concentrations significantly dropped after 24 h period of cystine-free feeding,. However, in adult humans fed a methionine adequate diet, a sulfur amino acid free diet or a methionine free but cystine supplemented diet, cystine plasma levels did not significantly change (63). A sulfur amino acid devoid diet reduces the flow of methionine via the transmethylation and transsulfuration pathways in subjects fed the test diet for five days (63). Infants in our study had an adaptation period of 24 h, which might have been too short for the methionine cycle to adjust to the induced cystine-free diet.

On the other hand, the group of Pencharz (28, 30) has demonstrated that 4 h of adaptation to the test amino acid level is sufficient to establish a new steady state. Since methionine is the precursor of cysteine, the methionine intake needs to be taken into account as well. The current estimated methionine requirement for preterm infants is 48-69 mg/(kg·d) (64). Shoveller et al. (65) determined the total sulfur amino acid requirement in enterally fed neonatal piglets when no dietary cysteine was provided. The extrapolation from this piglet study results in a methionine intake of 84 mg/(kg·d) for the human neonate, which is above the current recommendations (64). A very recent study has determined the total sulfur amino acid requirements in parenterally fed neonates to be 50 mg/(kg·d) (Courtney-Martin et al. in press AJCN 2008). Thus taken into account that the splanchnic methionine uptake is about 20%, the dietary intake should be approximately 70 mg/(kg·d). In our study, infants received a mean methionine intake of 74 mg methionine/(kg·d), which is in agreement with the above calculated quantity. It is slightly below the calculated requirement determined by Shoveller et al. but above the current recommended requirement for preterm infants (13). It might be, but seems unlikely, that the methionine intake in absence of cystine in our study was inadequate and that methionine disappeared in protein synthesis rather than that it entered into the transmethylation pathway.

In addition, we were not able to demonstrate endogenous cysteine synthesis in enterally fed preterm infants by incorporation of labeled serine into cysteine (**Chapter 6**). One explanation might be the route of tracer administration. In human studies investigating homocysteine metabolism, one carbon labeled serine was administered intravenously, but like in our study, no labeled cysteine could be detected (66-68). Even if two carbon atoms were labeled, no labeling of plasma cysteine could be measured (69). Eventually, the gastrointestinal tissues could preferentially utilize enteral serine for cysteine synthesis. We therefore hypothesized that the splanchnic tissues preferentially utilize serine derived from the diet rather than systemically derived serine for cysteine synthesis and for this reason we administered labeled serine enterally. But also during enteral administration, no net cysteine production could be measured. It might be possible that dietary serine was taken up by the splanchnic tissues in first-pass and utilized for other purposes, like conversion to glycine for the folate dependent transmethylation reaction. To optimize transsulfuration flux, infants were given a cystine-free but otherwise rich in protein diet. In contrast to the studies in human adults, our subjects were all studied in the postprandial state. The transsulfuration pathway is most active in the postprandial state when methionine is consumed (70).

Furthermore, it has been demonstrated that serine is the primary 1-C donor in the remethylation of homocysteine (66, 67, 71, 72). Approximately 80 to 90% of 1-C units is derived from the 3-C of serine. Consequently, glycine, histidine, betaine, sarcosine, and formate are minor sources of 1-C units for remethylation. Despite the strong dependence of the remethylation process on serine as a 1-C donor, only approximately 3% of the serine flux was used for remethylation in adults (66). Under our study conditions it is also possible that serine was predominantly used for conversion to glycine. Indeed, we found a significant amount of the serine carbon label back as [^{13}C]glycine. We could not detect plasma enrichment of [$^{13}C_4$]methionine in our infants (Chapter 6). The latter implies that if no dietary

cysteine is available, the remethylation is suppressed and likely all homocysteine is channeled directly towards the transsulfuration pathway. If this theory holds , we do not have a clear explanation for the absent cysteine enrichment. It is known that the isotopic enrichment of a nutritionally dispensable amino acid in plasma is lower than that for an equivalent tracer infusion of an indispensable amino acid because the pools of a dispensable are supplied not only by the diet but also from endogenous protein breakdown and the novo synthesis (73).

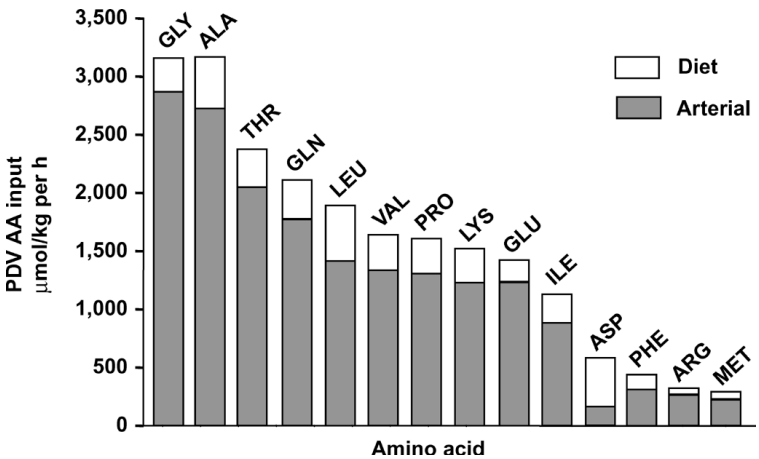
Two earlier studies have been attempted to investigate in vivo cysteine synthesis (74, 75). Preterm infants received uniformly labeled glucose intravenously to asses the endogenous cysteine synthesis from carbon atoms provided by glucose. In the first study in parenterally fed preterm infants supplemented with 86 mg cysteine/(kg·d), administration of uniformly labeled glucose did not yield labeled cysteine and the authors concluded that cysteine synthesis was insufficient and indispensable for these infants (74). The second study showed contradictory results by suggesting that the transsulfuration pathway is active in preterm infants receiving total parenteral nutrition (75). Following uniformly labeled glucose administration, cysteine enrichment was found in both plasma and apo B-100 but was very low, 0.45 and 1 mol% excess respectively. Again, both studies were performed in total parenterally fed preterm infants and no studies assessing the transsulfuration pathway have been performed in enterally fed infants. From the results in **chapter 3 and 4** we can conclude that the transsulfuration pathway is intact during enteral feeding and is likely to be ascribed to the intestinal cysteine synthesis. However, from the results in chapter 6, the transsulfuration pathway could not be determined to be active and came much to our surprise. Besides the route of feeding, this might be explained by the three different methods that were used to examine the endogenous cysteine synthesis. Shew et al. (75) were able to determine labeled cysteine by using a 4-h labeled glucose infusion, whereby glucose is converted to serine via 3-phosphoglycerate. In respect to this metabolic pathway it remains unexplained why Shew et al. could detect labeled cysteine and we could not. Perhaps ethnicity plays a role in sulfur amino acid metabolism.

Early *in vitro* animal studies showed that the methionine trans- and remethylation pathways are present in all tissues (76-78). In contrast, enzymes involved in the transsulfuration pathway (cystathionine ß-synthase (CBS) and cystathionine γ-lyase) are mainly active in liver tissue and to a lesser extent, in extra hepatic tissues including pancreas, kidney, small intestine, brain, and lung (54). Also, in preterm and term infants cystathionine ß-synthase is not only isolated to the liver but is present in the kidneys, adrenals, and, to a small degree in the pancreas as well (19). To emphasize the considerable role of the intestine in the transsulfuration pathway we have demonstrated the presence of cystathionine ß-synthase mRNA and its activity as well (**Chapter 5**). CBS mRNA expression and CBS activity was presented in liver, jejunum, stomach, pancreas and intestinal epithelial cells. The relative expression of CBS mRNA in liver tissue was > 10-fold higher than in any GI tissue measured. However, the CBS activity in liver tissue was only 3- to 5-fold higher than in gastrointestinal tissues. It is of interest that isolated epithelial cells had the highest CBS activity among the GI tissues measured. Oxidant stress is known to increase methionine transsulfuration to meet

the increased cysteine demand for cellular glutathione synthesis (79, 80). This suggests that the metabolic requirement for methionine and cysteine by the gut may be increased in conditions such as inflammatory bowel disease, enteric infection and in preterm infants.

SPLANCHNIC METHIONINE, ASPARTATE AND GLUTAMATE METABOLISM

The gut extensively metabolizes both dietary indispensable amino acids such as lysine, threonine, leucine, and dispensable amino acids, such as glutamate and aspartate (81-85). It is important to recognize that within the portal-drained-viscera, amino acids are presented via both the lumen and the arterial circulation. From a quantitative perspective, the supply of most amino acids from the arterial circulation is substantially greater (3- to 5-fold) than that



from the diet (Figure 2).

Figure 2. Amino acid input into portal-drained viscera (PDV) tissues from the diet and the arterial circulation in piglets. With permission, Journal of Animal Science 2006 (86).

However, the fractional portal-drained viscera use of dietary amino acids, ranging from 20 to 95%, is generally much greater than amino acids derived from the arterial blood, which ranges from approximately 5 to 15% (86). The reason for this observation is unclear, but may be due to differences in amino acid transporter abundance on the basolateral and apical surfaces of intestinal epithelial cells. Only a small number of studies have been performed to examine the site of amino acid absorption by the intestine. For example, in piglets glutamine is preferentially metabolized from the arterial site while glutamate is extensively taken up from the luminal site (87). The latter is likely due to the preferential source of enteral glutamate for mucosal glutathione synthesis (53). In piglets, dietary threonine is mostly used for protein synthesis in the small intestine (83), whilst the systemic threonine is used for synthesis of the intestinal mucus layer (Schaart, unpublished data). In addition, under normal feeding conditions, lysine and threonine used by the portal drained viscera derive almost entirely from the arterial site. We observed, contrary to our hypothesis, that the

gastrointestinal tissues in piglets preferentially utilized methionine from the arterial circulation rather than from the diet (**Chapter 5**). The arterial utilization of methionine indicates absence of first-pass intestinal metabolism of dietary methionine and might be explained by methodological considerations. Studies in adult humans fed a low–methionine but cystine supplemented diet showed comparable results (49, 50). Plasma enrichment of [1-^{13}C, methyl-$^{2}H_3$]methionine was similar during enteral and intravenous administration of the [1-^{13}C, methyl-$^{2}H_3$]methionine tracer. However, plasma enrichment of the derived methionine specie, [1-^{13}C]methionine, was considerably higher during enteral tracer administration. The remethylation and transmethylation rates were all higher when the enteral tracer was used than when an intravenous tracer was used. Thus, with a low maintenance intake of methionine, it appears that the free methionine pool undergoes significant methyl group transfer and remethylation during passage of dietary methionine through the splanchnic region, with an equivalent of the fraction of dietary methionine involved in this metabolic activity being released from the liver into the circulation. Likewise, our results showed significantly higher rates of both transmethylation and remethylation when the methionine tracers were administered enterally.

It has been well established, using various approaches, that splanchnic tissues metabolize a significant proportion of dietary amino acids in first-pass. In vivo studies in humans based on dual intravenous and intragastric administration of individual isotopic amino acid tracers, have shown that splanchnic tissues metabolize approximately 20–96% of the enterally administered amino acids (Figure 3) (50, 88-93). Rat studies showed that the three dispensable amino acids aspartate, glutamate and glutamine play a large role in intestinal energy metabolism (94). It is of great importance to investigate their contribution as an intestinal fuel in preterm infants within the scope of optimizing nutritional strategies.

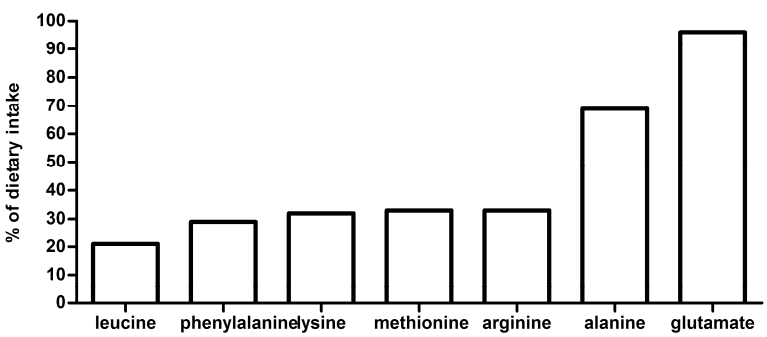


Figure 3. Splanchnic first-pass utilization of dietary amino acids in adults humans (50, 88-93).

Earlier studies performed in neonatal piglets showed that 20-50% of the dietary methionine was taken up by the gut in first-pass metabolism (82, 95). Additionally, the total sulfur amino acid requirement, as defined by the methionine requirement in absence of dietary cysteine, was 30% lower in parenterally, compared to enterally fed neonatal piglets (65). This difference suggests as well that methionine extraction by first-pass splanchnic metabolism

may be responsible. We also found that the intestine metabolizes an equivalent of 20% of the dietary methionine intake (**Chapter 5**) which is in agreement with previous studies.
In contrast to the widespread animal studies exploring in vivo methionine metabolism, not much is known about the methionine kinetics in preterm infants. We investigated the splanchnic methionine uptake in low-birth-weight infants (**Chapter 6**). In accordance with piglets studies we found that one third of the dietary methionine was taken up in splanchnic first-pass, which includes the liver, during the fed state. Interestingly, first-pass methionine uptake was absent in human studies during the fed state (50). In contrast, in the fasted state the methionine utilization varied from 25% for the low methionine-cysteine supplemented diet to 33% for the diet containing only methionine. This finding indicates that a considerable amount of methionine was used for cysteine synthesis. Surprisingly, in adults methionine uptake seems to appear at the basolateral site instead of the luminal site, which is inconsistent to our findings. In our study we fed infants a cyst(e)ine-free but containing an otherwise generous amounts of methionine to achieve optimal cyst(e)ine synthesis.

In contrast to methionine, aspartate and glutamate are indispensable amino acids for humans. In the early forties, several studies in rats already demonstrated that aspartate was a dispensable component for the diet (96, 97). Additional research was done to investigate the role of dietary glutamate and aspartate in rats and indicated that the addition of glutamate but not aspartate to a diet containing only the eight indispensable amino acids plus arginine and histidine, exerted a significant stimulatory effect on the rate of weight gain but that its removal from a diet containing dispensable and indispensable amino acids was followed by a very slightly inhibition of the weight gain (98).
Initially, glucose was thought to be the only respiratory fuel for the intestine (99). Studies in rats have shown that mucosal cells of the small intestine take up large amounts of arterial glutamine and that one third of CO_2 produced by intestine of fasted rats is derived from plasma glutamine and only 8% from glucose (100). A second source of metabolic substrates for intestinal mucosal cells, in addition to blood, is the gut lumen. To investigate the intestinal absorption of amino acids, an *in situ* method was developed and perfused intestinal loops of rats were examined (87, 94, 100-102). Virtually all glutamate and aspartate and significant amounts of glutamine and arginine absorbed from the lumen were metabolized. The resulting metabolic products were quantified and included CO_2, lactate, proline, citrulline, alanine, and glucose. The primary metabolic fate of luminal glutamate was oxidation to CO_2 (64%), followed by the formation to lactate (16%) and incorporation in proline, citrulline, ornithine and alanine (10%). Glutamate was found, as well as glutamine and glucose, to be an important contribution to energy requirements of the gut in rats (87, 100).
The gut of infant piglets metabolizes virtually all of the enteral glutamate (92%) during absorption (103). In human adults most of the dietary glutamate is metabolized in first-pass as well (88%) (104). In piglets also, glutamate is shown to be an important oxidative substrate for the intestinal mucosa (105). These studies in animals and adult humans suggest that dietary glutamate serves as an important metabolic fuel source in the intestine. Our study

investigating the splanchnic glutamate uptake and oxidation in preterm infants (**Chapter 7**) confirmed these observations. We found that approximately 75% of the dietary glutamate was taken up in splanchnic first-pass metabolism, which was slightly lower than observed in piglets and adult humans. Most of the extracted glutamate was channeled toward oxidation. This rate is comparable to the oxidation rate of glutamate in adults and indicates that this is the primary metabolic fate of dietary glutamate in the premature infant. Additionally, and in agreement with our results as well, a recent study demonstrated that the primary fate of dietary glutamate in premature infants is splanchnic extraction and not used for glucose production (106). Their findings, which indicate that dietary glutamate is not a significant gluconeogenic substrate, is not surprising, considering that we showed that oxidation is the primary metabolic pathway of enterally absorbed glutamate.

Apart from oxidation, glutamate can also act as a precursor for proline, arginine and glutathione. We were not able to determine the quantity of labeled alanine, lactate and pyruvate (**Chapter 7**). Thus, the contribution of $^{13}CO_2$ from oxidation from these products could not be quantified. However, based on animal data (87) we hypothesize that the contribution of labeled CO_2 from these amino acids is relatively small and that the fractional glutamate oxidation is not overestimated. Reeds et al (103) showed that in infant piglets infused with [U-^{13}C]glutamate [M+5] lower mass isotopomers appeared in plasma and determined the degree to which the ^{13}C-label was recycled in intermediary metabolism. They showed that 11% of the labeled glutamate appeared in [M+1] and [M+3] isotopomers. We, however, were not able to determine the present abundance of plasma [$^{13}C_3$]glutamate [M+3] since the [D_3]glutamate tracer has a mass of [M+3] as well and thus could not be distinguished from the [$^{13}C_3$]glutamate produced from intermediary metabolism.

Enterally derived glutamate is the preferential source for mucosal glutathione synthesis in neonatal piglets. Approximately 85% of the mucosal GSH-glutamate derived from dietary glutamate (53). If the intestine oxidizes almost all dietary glutamate, the quantity of remaining glutamate may be insufficient to maintain the preterm infant's demand for glutathione production during periods of stress, especially if parenteral feeding is needed. The minimal glutamate requirement for glutathione synthesis during stress and illness in human neonates is not yet known. Animal data, however, strongly indicate that mucosal GSH-glutamate is mainly derived from dietary glutamate. This could have implications for parenterally fed infants as they lack the supply of luminal (dietary) glutamate during parenteral feeding. The intestinal GSH synthesis may be restricted and therefore limiting protection against oxidative injury.

Further research revealed that besides glutamine and glutamate, aspartate was also a major fuel source for intestinal energy in rats (94). Indeed, virtually all aspartate was utilized in first-pass metabolism in neonatal piglets and rats (95, 102, 105). This phenomenon was also seen in enterally fed preterm infants, where 86% of the enteral aspartate was extracted in splanchnic first-pass metabolism **(Chapter 8).** In addition, it was determined in rats that more than 50% of the extracted aspartate was oxidized to CO_2 (102). Unfortunately, only in four infants whole-body and first-pass oxidation could be determined. In agreement to animal studies, three quarters of dietary aspartate uptake in first-pass was directed toward oxidation.

Like for glutamate, the major fate of dietary aspartate is uptake and oxidation within the splanchnic tissues in first-pass in preterm infants receiving full enteral feeding. Furthermore, almost 17% of the absorbed aspartate was recovered as lactate and 10% as alanine, indicating a second important role in three-carbon metabolism (102). Surprisingly, in rats 10% of the labeled aspartate was recovered as labeled glucose which is apparently the first demonstration of in vivo gluconeogenesis by the intestine (102). Due to methodological considerations it was not possible to measure incorporation of aspartate in labeled amino acids or organic acids as discussed previously for glutamate **(Chapter 8)**.

FUTURE PERSPECTIVES

Many topics related to the nutritional aspects in relation to the preterm infant are still subject for further research. The results obtained in this thesis can be used in future studies.
To explore the exact cysteine requirements for enterally fed preterm infants, it would be necessary to perform a study like described in Chapters 3 and 4 but instead using cyst(e)ine intakes between zero and 18 mg/(kg·d). The study should also be performed with varying methionine intakes because total sulfur amino acid requirement could be provided by solely methionine.
Furthermore, with the non-invasive IAAO method it is possible to chart the requirements of all indispensable amino acids in preterm and term infants which will allow us to further optimize nutrition. As discussed earlier, nitrogen balance was the primary method of choice to determine amino acid requirements. Until recently, recommendations were based on nitrogen balance studies. By the introduction of the carbon oxidation studies in human adults it became clear that the requirements of indispensable amino acids were much higher than previously assumed and the nitrogen balance estimates were in error by a factor 2 to 3 (30, 107-109). Recently, The Food and Nutrition Board of the Institute of Medicine (110) recommended the estimated requirements of human adults on carbon oxidation studies. The requirements are variable with time and also depend on the quality and quantity of ingested proteins. The quality of amino acids is an important determining factor for improving infant formula and more importantly, is cost-cutting. Establishing exact requirements of indispensable and dispensable amino acids will reduce the cost for manufacturing, since protein is the most expensive constituent in infant formula. In addition, it results in a diminished nitrogen load which will probably result in improvement of renal function. Reduced cost might lead to a reduction in price and subsequently an increased availability for developing countries. Several diseases and its related medications like for HIV, require formula to nourish infants and, therefore, the need for cheaper formula is continuously increasing.

In addition, the cysteine sparing capacity to reduce methionine requirements needs to be investigated. To date, animal and human studies have revealed different results on cysteine sparing capacity. Therefore, determination of minimal methionine requirements using

various methionine intakes in combination with variable additional cysteine intakes needs further investigation.
Another issue that needs further research is the endogenous cysteine synthesis in preterm infants. The results from the study in Chapter 6 might imply that there might be a minimal requirement for cysteine, although the plasma cysteine concentration is not a well chosen primary end-point. Adult studies have repeatedly failed to detect cysteine enrichment after administration of labeled serine. A modification of this method as described in this thesis has equally failed to reveal cysteine synthesis in preterm infants. Therefore, new methods to explore the transsulfuration pathway need to be designed and validated in neonatal piglets. Subsequently, this new method should be adapted to preterm infants. It is known that labeled homocysteine, formed from labeled methionine, takes approximately 6-8 h to reach a plateau (111). It is possible that the same counts for cysteine, especially after a period of cysteine free feeding whereby initially cysteine is released from glutathione, which is the primary cysteine storage. First, a pilot study should be performed using increasing infusion periods. Secondly, instead of determining enrichment of cysteine, cystathionine enrichment should be measured, which is formed by fusion of serine and homocysteine and occurs one step earlier in the transsulfuration pathway. Another suggestion is to make use of sulfur labeled methionine. However, this is currently not commercially available. In contrast, radioactive labeled sulfur methionine is available and using radioactive isotopic techniques to evaluate endogenous cysteine synthesis is suitable for animal studies. Next to metabolic pathways, it is possible to determine CBS enzyme activity in gut tissue taken from biopsy in animal studies or in patients undergoing intestinal surgery.
Furthermore, it needs to be established if the intestines preferentially utilize enteral or systemic cysteine for glutathione synthesis by use of stable isotope techniques. In our laboratory, we have developed a new method to measure glutathione in erythrocytes (112). In addition, it might be possible to collect biopsies to determine labeled glutathione in patients with an enterostomy. Enteral isotopic infusion should be compared to systemically infused labeled isotope.

With respect to glutamate and aspartate metabolism, additional research investigating the role of these amino acids in three carbon metabolism (alanine, pyruvate and lactate) will give further insight in intestinal metabolism of these amino acids. Moreover, it is important with regard to nutritional considerations, to determine minimal glutamate, cysteine and glycine requirements for glutathione synthesis during stress and illness in human neonates. Furthermore, it needs to be investigated if enteral glutamate is the main source for glutathione synthesis in these infants. If this holds true, this will have great implications in total parenterally fed infants but also during minimal enteral nutrition. The latter should therefore be supplemented with glutamate and the effect could be determined in a randomized controlled trial. The following primary endpoints could be used: glutathione synthesis rate and other oxidative stress markers, such as oxidized protein products (AOPP) and dityrosine. In addition, clinical features as the incidence of BPD and ROP could be determined.

It has been clearly established that enteral feeding has a major influence on short-term and long-term outcome in preterm infants. Nutritional requirements are higher in preterm infants that in term infants because of rapid growth. It is, therefore, of utmost importance to determine the estimated requirements of all dispensable amino acids in preterm infants to provide these infants balanced nutrition to optimize growth and outcome.

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Chapter 10

Summary
Samenvatting

SUMMARY

This dissertation describes the sulfur amino acid metabolism in the neonatal period. The results of both human and animal studies will be presented. It is divided into 4 parts.

In **Chapter 1** we describe the background and aims of the studies presented in this thesis. We give a general introduction to nutrition in preterm infants, such as the role of early nutrition, amino acids and their requirements. Furthermore, the role of sulfur amino acids and their requirements are discussed followed by intestinal metabolism.

Chapter 2 describes a study to validate the use of enteral infusion of isotopically labeled sodium bicarbonate in metabolic studies. Stable isotopes are often used in clinical studies to investigate amino acid metabolism. To determine the oxidation rate of the labeled substrates, the individual carbon dioxide production rate needs to be quantified. This is usually determined by intravenous infusion of labeled sodium bicarbonate, which requires a intravenous catheter. Placing an intravenous catheter is invasive in the enterally fed preterm infant who has no catheter and, therefore, we wish to reduce the invasiveness of the method that is used to determine individual carbon dioxide production. The aim of the study was to determine whether enteral and intravenous infusions of labeled sodium bicarbonate would achieve the same enrichment in expired breath. The second aim was to quantify the minimal tracer infusion time to achieve steady state during enteral infusion. We showed in 10 preterm infants that intravenous and intragastric labeled sodium bicarbonate yielded the same isotopic enrichment in expired breath. Secondly, the minimal enteral tracer infusion time required to establish steady state was 120 min. In conclusion, enteral infusion of labeled sodium bicarbonate may serve to estimate the carbon dioxide production rate in preterm infants and the infusion time is at least 120 min. We therefore administered the labeled sodium bicarbonate enterally in the cysteine requirement studies when infants were fully enterally fed.

Part 1 focuses primarily on the cyst(e)ine requirement for preterm infants.

In **Chapter 3**, we determined, one month after birth, the cyst(e)ine requirement in fully enterally fed preterm infants born with a gestational age of 32-34 wk. The fractional oxidation rate of [1-^{13}C]phenylalanine did not differ with increasing cyst(e)ine intakes. This result shows that cyst(e)ine was not the limiting amino acid for protein synthesis with an intake of at least 18 mg cyst(e)ine/(kg·d). Thus, in 4-wk-old preterm infants, born at gestational ages of 32-34 wk cyst(e)ine synthesis seems not limited. Therefore, it is probably not a conditionally essential amino acid in these infants. The minimal cyst(e)ine requirement for these infants is < 18 mg cyst(e)ine/(kg·d).

It is believed that the cyst(e)ine requirement depends on both gestational age and postmenstrual age. In **Chapter 4** we investigated the influence of gestational age as well as

the postmenstrual age on the cyst(e)ine requirement. We studied preterm infants who were born with a gestational age of < 29 wk both at postmenstrual ages of 32 and 35 wk. In the first group, fractional oxidation of [1-^{13}C]phenylalanine was determined ~ 1 month after birth (range: 31-32 wk postmenstrual age). In the second group, fractional oxidation of [1-^{13}C]-phenylalanine was determined ~ 2 months after birth (range: 35-38 wk postmenstrual age). In both groups, the fractional oxidation rates of [1-^{13}C]phenylalanine did not differ among the 5 graded cyst(e)ine intakes. The estimated minimal cyst(e)ine requirement in both groups was < 18 mg/(kg·d). From these results we can concluded that the cyst(e)ine requirement for preterm infants older than 32 wk postmenstrual age and who tolerate full enteral feeding is < 18 mg cyst(e)ine/(kg·d). The cyst(e)ine requirement does, therefore, not depend on postmenstrual age in enterally fed infants born with a gestational age < 29 wk. Thus, in enterally fed preterm infants born at gestational age < 29 wk the transsulfuration pathway seems active and cyst(e)ine synthesis not limited.

To answer the question if cyst(e)ine requirement depends on gestational age we need to compare the results from **Chapter 3** and **Chapter 4**. In Chapter 3 we determined the cyst(e)ine requirement in preterm infants who were born with a gestational age of 32-34 wk and in Chapter 4, preterm infants were born with a gestational age of < 29 wk. Thus, we compared two groups with different gestational ages but at the same postmenstrual age. The cyst(e)ine requirement of both groups did not differ and was < 18 mg cyst(e)ine/(kg·d). Therefore cyst(e)ine requirement does not depend on either gestational age nor postmenstrual age.

Part 2 focuses on intestinal sulfur amino acid metabolism in neonatal piglets and preterm infants during complete enteral feeding.

Chapter 5 describes the metabolic fate of methionine in the gastrointestinal tissues (GIT) of neonatal pigs. We found that the GIT metabolize an equivalent of ~ 20% of the dietary methionine intake. Whole-body methionine transmethylation to homocysteine and remethylation of homocysteine to methionine was higher during enteral tracer infusion than intravenous infusion, and indicates that there was specific first-pass metabolism of methionine. The most important finding is that ~2/3 of the methionine taken up by the GIT was used for homocysteine and cysteine synthesis. Thus, the GIT is indeed an important site for sulfur amino acid metabolism and represents 27% and 23% of whole-body rates of transmethylation and transsulfuration, respectively.
Furthermore, we demonstrated cystathionine ß-synthase (CBS) mRNA expression and enzyme activity in various GIT (liver, jejunum, stomach, pancreas and intestinal epithelial cells). The CBS mRNA expression was highest in liver tissue. Interestingly, the CBS activity was highest in intestinal epithelial cells among the tissues measured. Activity increased 6-fold after incubation with S-adenosylmethionine, which is an allosteric activator of CBS.

After the results obtained in neonatal piglets, we performed a similar stable isotope study in

preterm infants in **Chapter 6**. In this study we quantified the splanchnic methionine first-pass uptake in preterm infants with a mean gestational age of 32 wk. Furthermore, we examined endogenous cysteine synthesis by incorporation of enterally administered labeled serine into cysteine. To increase the transsulfuration activity, infants were fed a cyst(e)ine-free diet for 24 h. Plasma methionine concentrations did not differ after 24 h of cyst(e)ine-free feeding. However, plasma cystine concentrations dropped significantly from 37 μmol/L to 19 μmol/L. This decrease in plasma cystine concentration after a cyst(e)ine-free diet implies that cyst(e)ine consumption exceeded endogenous synthesis. This is in contrast with the results shown in Chapter 3 and 4, where we concluded that the cysteine synthesis pathway was sufficient. Furthermore, we demonstrate that 35% of the dietary methionine intake was taken up in first-pass metabolism. We were not able to detect the serine carbon label in cysteine. However, we found a significant enrichment in plasma glycine derived from labeled serine. These findings indicate that under the conditions of enteral feeding and a cyst(e)ine-free diet enteral serine is not used for endogenous cysteine synthesis.

Part 3 focuses on splanchnic metabolism of the nonessential amino acids glutamate and aspartate in preterm infants.

In **Chapter 7**, we studied whole-body and splanchnic first-pass glutamate metabolism in preterm infants. We quantified first-pass glutamate uptake and splanchnic oxidation in infants with a mean gestational age of 29 wk. As expected, the fractional first-pass uptake of glutamate by the splanchnic tissues was high. Approximately three quarters of the enteral intake was sequestered by the splanchnic tissues. Furthermore, almost all (86%) was directed toward oxidation.

Finally, in addition to the previous chapter, we investigated splanchnic aspartate metabolism in fully enterally fed preterm infants in **Chapter 8**. Like glutamate, first-pass aspartate uptake by the splanchnic tissues was almost complete and amounted 77%. More than three quarters of the aspartate used in first-pass was directed toward oxidation. The results of Chapter 7 and 8 indicate that the primary metabolic fate of the nonessential amino acids glutamate and aspartate is splanchnic oxidation.

Part 4 presents **Chapter 9**, where we discuss our findings. Furthermore, we make recommendations for future studies. In **Chapter 10** all studies presented in this thesis are summarized.

The main conclusions obtained from the studies described in this thesis are the following:

- The cysteine requirement for one-month-old enterally fed preterm infants born with gestational age < 29 wk is < 18 mg cyst(e)ine/(kg·d).

- The cysteine requirement for preterm infants does not depend on gestational and

postmenstrual age in one-month-old enterally fed infants (> 32 wk postmenstrual age) who are born with a gestational age < 29 wk.

- The gastrointestinal tissues are an important site for both homocysteine and cysteine synthesis.

- Plasma cystine concentration decreases after a period of cysteine-free feeding in preterm infants with a mean gestational age of 32 wk. This finding implies that either cyst(e)ine consumption exceeded endogenous synthesis or that enzyme activity for cysteine synthesis might not be fully developed.

- Enterally administered labeled serine was not used for endogenous cysteine synthesis in preterm infants fed a cysteine-free diet. Other metabolic pathways for cysteine synthesis may exist.

- Glutamate and aspartate are important energy sources for splanchnic tissues in preterm infants.

SAMENVATTING

Inleiding

De zwavelhoudende aminozuren zijn: methionine, homocysteine en cysteine. Cyst(e)ine wordt gebruikt om elke onduidelijke combinatie van cystine en cysteine aan te duiden.

Te vroeg geboren kinderen hebben na de geboorte een hoge behoefte aan voedingsstoffen en calorieën als gevolg van aanpassingen aan de extra-uteriene omgeving en door onrijpheid. Als gevolg van de vroeggeboorte zijn sommige metabole pathways nog niet volledig ontwikkeld. Dit heeft als gevolg dat sommige niet-essentiële aminozuren tijdelijk essentieel zijn; dit wil zeggen dat het lichaam niet in staat is om aan de minimale behoefte van dit aminozuur te voldoen. Deze aminozuren moeten dan tijdelijk in voldoende mate worden gesuppleerd in de voeding om aan de behoefte te voldoen. Daarnaast verschilt de aminozuurbehoefte van te vroeg geboren kinderen ten opzichte van a terme geboren kinderen als gevolg van een hogere groeisnelheid. De gewichtstoename van te vroeg geboren kinderen is na de geboorte een stuk lager in vergelijking met de foetus met dezelfde zwangerschapsduur in de baarmoeder. Om een vergelijkbare groeisnelheid als in utero te bereiken, is juiste en gebalanceerde voeding noodzakelijk. Het is daarom belangrijk om de minimale behoefte van essentiële aminozuren van te vroeg geboren kinderen te bepalen.

Gedacht wordt dat cyst(e)ine tijdelijk een essentieel aminozuur is in te vroeg geboren kinderen als gevolg van onvoldoende cyst(e)ine productie (synthese). De cyst(e)ine synthese wordt gereguleerd door het enzym cystathionase. Studies uit de jaren 70 hebben laten zien dat de activiteit van het enzym cystathionase verminderd is en voornamelijk plaatsvindt in de lever. Het is mogelijk dat de maximale enzymcapaciteit afhankelijk is van zowel de zwangerschapsduur en de postnatale leeftijd. Het is dus van belang om de minimale cyst(e)ine behoefte te bepalen in te vroeg geboren kinderen, echter dit is nog niet door middel van klinische studies vastgesteld.

Daarnaast wordt gedacht dat naast de lever de darm een belangrijke rol speelt bij de cyst(e)ine synthese. De darm heeft verschillende functies, waaronder digestie en absorptie van voedingstoffen. Aminozuren worden door de darm in first-pass opgenomen en gebruikt voor onder andere de synthese van (glyco)proteïnen, voor tussenliggend metabolisme of voor oxidatie. Dit laatste resulteert in het vrijkomen van energie. Uit dierstudies is gebleken dat naast glucose, de niet essentiële aminozuren glutamaat en aspartaat een belangrijke rol spelen in de energie voorziening van de darm.

Dit proefschrift beschrijft het metabolisme van zwavelhoudende aminozuren in de neonatale periode en bestaat uit vier delen. De resultaten van een diermodel studie en klinische patiënten studies worden beschreven. In **deel 1** wordt de minimale behoefte van cyst(e)ine in te vroeg geboren kinderen met verschillende zwangerschapsduur en op verschillende postnatale leeftijd beschreven. In **deel 2** wordt het metabolisme van zwavelhoudende

aminozuren in de darm en in het splanchnisch gebied beschreven. Vervolgens wordt in **deel 3** het splanchnisch metabolisme van twee niet essentiële aminozuren in te vroeg geboren kinderen beschreven. In **deel 4** worden de verschillende hoofdstukken samengevat en bediscussieerd.

In **hoofdstuk 1** worden de achtergronden en doelstellingen van de in dit proefschrift gepresenteerde studies beschreven. Het belang van voeding bij te vroeg geboren kinderen en de behoefte van aminozuren en het bepalen daarvan worden beschreven. Vervolgens wordt ingegaan op de zwavelhoudende aminozuren en aminozuur metabolisme van de darm.

Hoofdstuk 2 beschrijft een onderzoek die als doel heeft om een bestaande methode die de koolstofdioxide (CO_2) productie in kinderen meet eenvoudiger te maken. In studies die het metabolisme van aminozuren onderzoeken is het noodzakelijk om de individuele stofwisseling te bepalen. Dit gebeurt door middel van een stabiel isotoop via het infuus toe te dienen en vervolgens de hoeveelheid CO_2 in de uitademinglucht te bepalen. Bij te vroeg geboren kinderen is het echter belastend om een infuus in te brengen indien dit enkel voor het onderzoek noodzakelijk is. Daarom is de huidige methode vergeleken met het toedienen van het stabiele isotoop via een maagsonde. De opbrengst van CO_2 in de uitademinglucht was voor beide methoden gelijk. Uit dit onderzoek mag dus geconcludeerd worden dat de intraveneuze infusie van het stabiele isotoop kan worden vervangen door enterale infusie om de individuele CO_2 productie te bepalen. De nieuwe methode om de CO_2 productie te bepalen is dan ook in dit proefschrift gebruikt.

Deel 1. Minimale cyst(e)ine behoefte in te vroeg geboren kinderen.

In **hoofdstuk 3** is gekeken naar de minimale behoefte van het aminozuur cyst(e)ine in te vroeg geboren kinderen die geboren werden met een zwangerschapsduur van 32 tot 34 weken (gemiddeld 33 weken). De cyst(e)ine behoefte werd ongeveer een maand na de geboorte bepaald, op de postmenstruele leeftijd van 36 weken. De minimale behoefte bleek minder dan 18 mg/(kg·d) cyst(e)ine te bedragen. Ter vergelijking, standaard kunstvoeding voor te vroeg geboren kinderen bevat 54 mg/(kg·d) cyst(e)ine. Dit betekent wellicht dat te vroeg geboren kinderen op de leeftijd van een maand in staat zijn om voldoende cyst(e)ine te produceren om aan de behoefte te voldoen. Hieruit kan indirect worden vastgesteld dat cyst(e)ine waarschijnlijk geen tijdelijk essentieel aminozuur is in een maand oude kinderen met volledig enterale voeding die geboren zijn met een zwangerschapsduur van 32 tot 34 weken.

Uit eerdere studies is gebleken dat de activiteit van het enzym cystathionase mogelijk ook afhankelijk is van de leeftijd na de geboorte. In **hoofdstuk 4** wordt vervolgens onderzocht of er een relatie bestaat tussen de postnatale leeftijd en de cyst(e)ine behoefte van te vroeg geboren kinderen. In kinderen geboren met een zwangerschapsduur van < 29 weken

(gemiddeld 27 weken) wordt de cyst(e)ine behoefte bepaald ongeveer een maand na de geboorte (gemiddelde postmenstruele leeftijd van 32 weken) en ongeveer twee maanden na de geboorte (gemiddelde postmenstruele leeftijd van 35 weken). Alle kinderen werden volledig enteraal gevoed. De behoefte bleek op beide leeftijden < 18 mg cyst(e)ine/(kg·d) te zijn. Hieruit kan geconcludeerd worden dat de cyst(e)ine behoefte van kinderen die geboren zijn met een zwangerschapsduur < 29 weken die volledig enteraal gevoed worden, niet afhankelijk is van de postnatale leeftijd.

Tevens bestaat de idee dat de activiteit van het enzym cystathionase afhankelijk is van de zwangerschapsduur. De cyst(e)ine behoefte van te vroeg geboren kinderen die geboren werden met een zwangerschapsduur van < 29 weken (hoofdstuk 4) werd vergeleken met de behoefte van kinderen die geboren werden met een zwangerschapsduur van 33 weken. De behoefte werd in de twee groepen ongeveer een maand na de geboorte bepaald en bedroeg in beide groepen < 18 mg cyst(e)ine/(kg·d) en is dus niet verschillend. Hieruit mag worden afgeleid dat in 1 maand oude kinderen geboren met een zwangerschapsduur van 27 weken onder de omstandigheden van volledig enterale voeding de cyst(e)ine behoefte niet afhankelijk is van de zwangerschapsduur noch van de postnatale leeftijd.

Deel 2. Metabolisme van zwavelhoudende aminozuren in de darm en splanchnisch gebied.

In dit deel wordt ingegaan op de rol van de darm in de synthese van cysteine. Aangenomen wordt dat de cysteine productie met name in de lever plaatsvindt. Echter, er zijn aanwijzingen dat de darm hier ook een grote bijdrage levert. In **hoofdstuk 5** wordt het nut van methionine in de darm en de cysteine synthese door de darm van neonatale biggen onderzocht. Ongeveer twee derde van de methionine die wordt opgenomen door de darm werd gebruikt voor de synthese van homocysteine en cysteine. Dus de darm speelt inderdaad een belangrijke rol in de cysteine synthese. De bijdrage van de darm aan de homocysteine en cysteine synthese ten opzichte van totale lichaamshoeveelheden zijn respectievelijk ~30% en ~25%. Tevens wordt de mRNA expressie van het enzym cystathionase aangetoond in verschillende gastrointestinale weefsels, zoals lever, dunne darm, maag en darmepitheel. De expressie van mRNA bleek het hoogst in de lever. De activiteit van het enzym was het hoogst in de lever, gevolgd door darmepitheel.

Na de resultaten verkregen in neonatale biggen is een vergelijkbare studie gedaan in te vroeg geboren kinderen. In **hoofdstuk 6** wordt het metabolisme van zwavelhoudende aminozuren in volledig enteraal gevoede te vroeg geboren kinderen beschreven. Er is gebruikt gemaakt van verschillende stabiel isotoop gelabelde methionine tracers en een serine tracer die simultaan intraveneus en enteraal werden toegediend. Ongeveer een derde van de enterale methionine wordt verbruikt in first-pass metabolisme door het splanchnisch gebied (lever en darm). Vervolgens werd getracht om de synthese van cysteine met behulp van de isotoop gelabelde tracers aan te tonen. Echter, in deze studie kan de endogene synthese van cysteine uit methionine en serine niet worden aangetoond. Wel is aangetoond

dat de enterale serine werd gebruikt voor de omzetting naar een ander aminozuur namelijk glycine. Het einddoel en de verdere metabole route kon niet worden bepaald en zal verder moeten worden uitgezocht.
Tevens werd er een significante daling van de cystine concentratie in het plasma vastgesteld na een cyst(e)ine vrij dieet gedurende 24 uur. Deze bevinding suggereert dat het cyst(e)ine verbruik de endogene synthese overschrijdt. Een andere mogelijke verklaring is dat de synthese niet voldoet aan de behoefte en dat in te vroeg geboren kinderen de cyst(e)ine behoefte tussen de 0 en 18 mg/(kg·d) ligt.

Deel 3. Metabolisme van de niet essentiële aminozuren glutamaat en aspartaat in het splanchnisch gebied.

Dierstudies laten zien dat de darm de niet essentiële aminozuren glutamaat en aspartaat in grote hoeveelheden als energiebron gebruikt. In **hoofdstuk 7** worden de resultaten van splanchnisch- en totaal lichaamsmetabolisme van glutamaat in te vroeg geboren kinderen gepresenteerd. Het doel was om te bepalen hoeveel van de glutamaat in de voeding er door het splanchnisch gebied wordt opgenomen en vervolgens wordt geoxideerd (verbrand). Ongeveer tweederde van de enterale glutamaat inname wordt verbruikt in first-pass door het splanchnisch gebied in prematuur geboren kinderen. Vervolgens wordt merendeel (86%) hiervan geoxideerd door de darm. Glutamaat wordt dus met name gebruikt voor oxidatie in het splanchnisch gebied. In aansluiting hierop wordt in **hoofdstuk 8** het metabole einddoel van enteraal aspartaat in te vroeg geboren kinderen bestudeerd. Uit deze studie blijkt dat de darm, evenals glutamaat, driekwart van het aspartaat uit de voeding opneemt. Daarvan wordt tweederde gebruikt voor oxidatieve processen. Uit de resultaten van hoofdstuk 7 en 8 mag worden gesteld dat de niet essentiële aminozuren aspartaat en glutamaat uit de voeding belangrijke brandstoffen zijn voor het splanchnisch gebied in te vroeg geboren kinderen.

In **hoofdstuk 9** bevat een algemene discussie van de resultaten en met suggesties voor vervolg onderzoek.

De belangrijkste conclusies die uit de studies beschreven in dit proefschrift kunnen worden getrokken, zijn:

- De cyst(e)ine behoefte in enteraal gevoede te vroeg geboren kinderen die geboren zijn met een zwangerschapsduur < 29 weken is < 18 mg/(kg·d).

- In enteraal gevoede prematuren die geboren zijn met een zwangerschapsduur van < 29 weken is de cyst(e)ine behoefte niet afhankelijk van de zwangerschapsduur en de postnatale leeftijd.

- De gastrointestinale weefsels leveren een substantiële bijdrage aan de homocysteine

en cysteine synthese.

- In te vroeg geboren kinderen daalt de cyst(e)ine concentratie in het plasma aanzienlijk ten tijde van een cyst(e)ine vrij dieet.

- In te vroeg geboren kinderen wordt de serine uit de voeding niet gebruikt voor de cyst(e)ine synthese in het lichaam tijdens een cyst(e)ine vrij dieet.

- Glutamaat en aspartaat uit de voeding zijn belangrijke energiebronnen voor de darm in te vroeg geboren kinderen.

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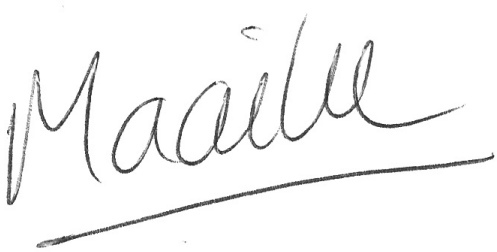
En de rest….. to be continued…….



CURRICULUM VITAE

Maaike Riedijk was born on April 28th, 1976 in Almelo, the Netherlands. She received secondary education at the KSG de Breul in Zeist and passed her VWO exam in 1995. In the same year she started her medical training at the faculty of Medicine at the University of Utrecht. During her study she did a clerkship at the department of surgery in Copenhagen, Denmark did a clinical rotation at the Massachusetts Eye and Ear Infirmary, Boston, USA.

From September 1999 until June 2000 she participated in a project investigating the exercise induced complications of meningococcal septic shock syndrome at the department of Pediatric Intensive Care Unit, Wilhelmina Children's Hospital, University of Utrecht, the Netherlands. During the fall of 2000, she participated in a project on burn-induced immunosuppression in a mouse model at the Centre des Grands Brûlés, Centre de Recherche, Centre Hospitalier de l'Université de Montréal, Hôtel-Dieu, Montréal, Quebec, Canada.

After obtaining het medical degree in 2002, she worked as an assistant in a general practice. In February 2003 she started as a research fellow at the department of Pediatrics, division of Neonatology at the Erasmus MC-Sophia Children's Hospital in Rotterdam (supervised by Prof. dr. J.B. van Goudoever), working on the research presented in this thesis. As part of her research project she worked from September 2003 until July 2004 in the laboratory at the Children's Nutrition Research Center, Baylor College of Medicine, Houston, USA (supervised by Dr. D.G. Burrin). She also performed a clinical study in Children's Hospital, Fudan University, Shanghai, China P.R. (supervised by Dr. Y. Huang) from October 2006 until December 2006.

During her research she participated in the board of residents of the Erasmus MC, Rotterdam and organized the Research Day of the Erasmus MC 2006 and 2008.
In April 2007 she started as a resident (ANIOS) at the Erasmus MC-Sophia Children's Hospital, Rotterdam and from November 2007 onwards she started her clinical pediatric residency in training (AIOS) here (heads, Prof. dr. A.J. van der Heijden and Dr. M. de Hoog).

During her research training she ran two marathons (Berlin marathon 2006, Rotterdam marathon 2007) and several half marathons (eg, Shanghai marathon 2006). She lives together with Rogier de Jonge in Utrecht.

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