Methionine requirement of the enterally fed term infant in the first month of life in the presence of cysteine1–3

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ABSTRACT

Background: The essential amino acid methionine can be used for protein synthesis but also serves as a precursor for homocysteine and cysteine.

Objective: The objective of this study was to determine the minimal obligatory methionine requirement of infants in the presence of excess cysteine (91 mg · kg⁻¹ · d⁻¹) by using the indicator amino acid oxidation (IAAO) method with L-[1-13C]phenylalanine as the indicator.

Design: Fully enterally fed term infants <1 mo of age were randomly assigned to methionine intakes that ranged from 3 to 59 mg · kg⁻¹ · d⁻¹ as part of an elemental formula. After 1 d of adaptation to the test diet, [¹³C]bicarbonate and L-[1-¹³C]phenylalanine tracers were given enterally. Breath samples were collected at baseline and during isotopic plateaus. The mean methionine requirement was determined by using biphasic linear regression crossover analysis on the fraction of F¹³CO₂ recovery from L-[1-¹³C]phenylalanine oxidation (F¹³CO₂). Data are presented as means ± SDs.

Results: Thirty-three neonates (gestational age: 39 ± 1 wk) were studied at 13 ± 6 d. With increasing methionine intakes, F¹³CO₂ decreased until a methionine intake of 38 mg · kg⁻¹ · d⁻¹; additional increases in methionine intake did not affect F¹³CO₂. The mean methionine requirement was determined at 38 mg · kg⁻¹ · d⁻¹, and the upper and lower CIs were 48 and 27 mg · kg⁻¹ · d⁻¹, respectively (P < 0.0001, r² = 0.59).

Conclusions: Although the current recommended methionine intake of 28 mg · kg⁻¹ · d⁻¹ is within the CIs of our study, the estimated mean requirement is substantially higher. However, most of the infant formulas provide a methionine intake of 49–80 mg · kg⁻¹ · d⁻¹, which is above the upper CI of our study. This trial was registered at www.trialregister.nl as NTR1610. Am J Clin Nutr 2012;95:1048–54.

INTRODUCTION

Methionine is an essential amino acid required for protein synthesis. It is also needed for the biosynthesis of carnitine, which is essential for fatty acid metabolism (1). Methionine is the major methyl donor in mammalian cells and a precursor for polyamine synthesis (2). The transmethylation of methionine leads to homocysteine synthesis. Homocysteine can be remethylated to form methionine or catabolized via the transsulfuration pathway to form cysteine. Cysteine can be incorporated into protein and is also involved in the production of glutathione, taurine, CoA, and inorganic sulfur. Cysteine, glutathione, and taurine play a role in the defense mechanism against oxidative stress.

A deficient intake of methionine not only impairs growth but has also an impact on the sulfur metabolic pathways in the synthesis of its key metabolic intermediates. In contrast, methionine is known as the most toxic amino acid in animals when supplemented in excess (3, 4). Hypermethioninemia and hyperhomocysteinemia were observed in infants who consumed a methionine-fortified formula with a methionine content of 788 mg/L or a high-protein formula that provided 9 g protein · kg⁻¹ · d⁻¹ (5, 6). Extreme hypermethioninemia may cause cerebral edema (5). Hyperhomocysteine has been shown to be associated with increased risk of neonatal stroke (7). Because both a deficient and excess intake of methionine have detrimental effects, it is important to determine the methionine requirement to optimize infant nutrition.

Experimental evidence of the methionine requirement of enterally fed infants is scarce. In previous studies with a relatively small number of infants (n = 7–13), the methionine requirement was estimated to be between 27 and 49 mg · kg⁻¹ · d⁻¹ (8–11). Because breast milk is considered to be the optimal nutrition for infants ≤6 mo of age, the joint WHO/FAO/UNU expert consultation recommended a methionine intake of 28 mg · kg⁻¹ · d⁻¹ on the basis of the average intake of breastfed infants (12). Human milk is known to vary in protein content, whereas the volume ingested also varies on a daily basis. These factors all contribute

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2 Sponsored by Danone. Study formulas were manufactured by SHS UK, and transportation to Shanghai was facilitated by Dumex China. The study sponsors had no influence on the study design, the analysis of the data, or the writing of this manuscript.

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to the difficulty of providing an accurate estimation of the intake of a breastfed infant.

The indicator amino acid oxidation (IAAO) method is minimally invasive and, therefore, suitable for the determination of the essential amino acid requirements in vulnerable populations including infants (13, 14).

The aim of this study was to determine the minimal obligatory methionine requirement with an excess intake of cysteine of term infants by using the IAAO method.

SUBJECTS AND METHODS

Subjects

Thirty-three neonates admitted to the Neonatal Ward in the Children’s Hospital of Fudan University participated in the study. Each subject was selected for study by using the following criteria: fully enterally fed infants with a gestational age ≥37 wk, birth weight ≥2500 g, and clinically stable with a weight-gain rate ≥5 g · kg⁻¹ · d⁻¹ in the preceding 3 d. Subjects were excluded if they had congenital anomalies, gastrointestinal pathology, or sepsis.

The study was approved by the institutional review boards of the Children’s Hospital of Fudan University, and a statement of no intestinal pathology, or sepsis.

The study design was based on the minimally invasive IAAO method (13), which was recently modified to apply in enterally fed infants (14). The advantages of this method are the short adaptation period to the test intake (1 d), the enterally delivered isotopes, and the sampling of expired air without sampling of the amino acid content. Methionine, which was completely withdrawn from the study diet on day 1. An equivalent amount of L-phenylalanine (0.52 g/100 g formula) was given as isotope on day 2.

by the substitution of L-alanine for the methionine that was withdrawn. The caloric intake was kept constant during the study period in all infants.

The minerals and trace elements supplied in 100 g formula were as follows—iron: 7.0 mg; calcium: 325 mg; phosphorus: 230 mg; magnesium: 34 mg; sodium: 120 mg; chloride: 290 mg; potassium: 420 mg; manganese: 0.38 mg; iodine: 47 µg; selenium: 11 µg; copper: 380 µg; and zinc: 5.0 mg.

The vitamin content of 100 g formula was as follows—vitamin A: 528 µg retinol equivalent; vitamin D: 8.5 µg; vitamin E: 3.3 mg α-tocopherol equivalent; vitamin K: 21 µg; thiamine: 390 µg; riboflavin: 600 µg; niacin: 4.5 mg; vitamin B-6: 520 µg; vitamin B-12: 1.3 µg; pantothenic acid: 2.3 mg; folic acid: 38 µg; vitamin C: 40 mg; and biotin: 26 µg.

Experimental design

The study design was based on the minimally invasive IAAO method (13), which was recently modified to apply in enterally fed infants (14). The advantages of this method are the short adaptation period to the test intake (1 d), the enterally delivered isotopes, and the sampling of expired air without sampling of the amino acid enrichments in plasma or urine. The IAAO method is based on the concept that, when the test amino acid intake is insufficient to meet the requirement, protein synthesis will be limited and all of the amino acids will be oxidized, including the indicator amino acid, which is labeled with a stable isotope. As the dietary intake of the

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**TABLE 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 100 g formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>475</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>54</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>23</td>
</tr>
<tr>
<td>Total amino acid (g)</td>
<td>10</td>
</tr>
<tr>
<td>l-Alanine (g)⁴</td>
<td>≥0.61</td>
</tr>
<tr>
<td>l-Arginine (g)</td>
<td>1.08</td>
</tr>
<tr>
<td>l-Asparagine (g)</td>
<td>1.01</td>
</tr>
<tr>
<td>l-Cysteine (g)</td>
<td>0.4</td>
</tr>
<tr>
<td>Glycine (g)</td>
<td>0.95</td>
</tr>
<tr>
<td>l-Histidine (g)</td>
<td>0.62</td>
</tr>
<tr>
<td>l-Isoleucine (g)</td>
<td>0.95</td>
</tr>
<tr>
<td>l-Leucine (g)</td>
<td>1.63</td>
</tr>
<tr>
<td>l-Lysine (g)</td>
<td>1.11</td>
</tr>
<tr>
<td>l-Methionine (g)⁵</td>
<td>0</td>
</tr>
<tr>
<td>l-Phenylalanine (g)⁶</td>
<td>0.20</td>
</tr>
<tr>
<td>l-Proline (g)</td>
<td>1.16</td>
</tr>
<tr>
<td>l-Serine (g)</td>
<td>0.71</td>
</tr>
<tr>
<td>l-Threonine (g)</td>
<td>0.8</td>
</tr>
<tr>
<td>l-Tryptophan (g)</td>
<td>0.32</td>
</tr>
<tr>
<td>l-Tyrosine (g)</td>
<td>0.73</td>
</tr>
<tr>
<td>l-Valine (g)</td>
<td>1.04</td>
</tr>
<tr>
<td>l-Carnitine (g)</td>
<td>0.01</td>
</tr>
<tr>
<td>Taurine (g)</td>
<td>0.03</td>
</tr>
<tr>
<td>l-Glutamine (g)</td>
<td>1.34</td>
</tr>
</tbody>
</table>

¹ Variable amounts of l-alanine were added to the diet depending on the test methionine amount of each infant to maintain an isonitrogenous diet. The study formula contained ≥0.61 g l-alanine/100 g formula.

² l-Methionine was added separately depending on the test methionine amount.

³ At total of 0.53 g l-phenylalanine/100 g formula was added to the study diet on day 1. An equivalent amount of l-phenylalanine (0.52 g/100 g formula) was given as isotope on day 2.

⁴ Abbreviations used: APE, atom percentage excess; F13CO2, fraction of 13CO2 recovery from l-[1-13C]phenylalanine oxidation; IAAO, indicator amino acid oxidation.
test amino acid increases, the oxidation rate of the indicator will decrease until the requirement of the test amino acid is met. Once the requirement of the test amino acid is met, an additional increase in its intake will have no additional influence on the oxidation rate of the indicator amino acid. The oxidation of the indicator amino acid can be measured in expired air as $^{13}$CO$_2$.

During the study, all infants received a fluid intake of $\sim$150 mL·kg$^{-1}$·d$^{-1}$, a caloric intake of 108 kcal·kg$^{-1}$·d$^{-1}$, and an amino acid intake equal to the protein intake of $\sim$2.96 g·kg$^{-1}$·d$^{-1}$. Infants were randomly assigned to one of the graded test intakes of methionine, which ranged from 3 to 59 mg·kg$^{-1}$·d$^{-1}$. To maximize the power of the breakpoint analysis used in our method, a wide range of methionine intakes with approximately equal number of intakes above and below the expected breakpoint was chosen (16). Because the methionine requirement was expected to approximate the methionine content in human milk, we studied an equal number of intakes above and below the expected requirement of 28 mg·kg$^{-1}$·d$^{-1}$ (12). Each study took place over a 31-h period, whereby each infant received one of the test intakes. After 24 h of consumption of the study formula, tracers were administered on day 2 for 7 h. In its intake will have no additional influence on the oxidation rate of the indicator amino acid. The oxidation of the indicator amino acid can be measured in expired air as $^{13}$CO$_2$. During the study, all infants received a fluid intake of $\sim$150 mL·kg$^{-1}$·d$^{-1}$, a caloric intake of 108 kcal·kg$^{-1}$·d$^{-1}$, and an amino acid intake equal to the protein intake of $\sim$2.96 g·kg$^{-1}$·d$^{-1}$. Infants were randomly assigned to one of the graded test intakes of methionine, which ranged from 3 to 59 mg·kg$^{-1}$·d$^{-1}$. To maximize the power of the breakpoint analysis used in our method, a wide range of methionine intakes with approximately equal number of intakes above and below the expected breakpoint was chosen (16). Because the methionine requirement was expected to approximate the methionine content in human milk, we studied an equal number of intakes above and below the expected requirement of 28 mg·kg$^{-1}$·d$^{-1}$ (12). Each study took place over a 31-h period, whereby each infant received one of the test intakes. After 24 h of consumption of the study formula, tracers were administered on day 2 for 7 h. Infants were bottle-fed every 3 h during the adaptation period. Subsequently, the feeding regimen changed to an hourly bottle-feeding during the tracer infusion until the end of the study. On the tracer day, a nasogastric tube was placed for tracer infusion. Infants received a primed (14·mol/kg) continuous (9·mol·kg$^{-1}$·h$^{-1}$) enteral infusion of $^{13}$C bicarbonate (sterile pyrogen free, 99% $^{13}$C atom percentage excess (APE; Cambridge Isotopes) for 3 h to quantify individual CO$_2$ production rates (17). Phenylalanine was used as the indicator. After the $^{13}$C bicarbonate infusion was stopped, a primed (34·mol/kg) continuous (27·mol·kg$^{-1}$·h$^{-1}$) enteral infusion of L-[1-$^{13}$C]phenylalanine (99% $^{13}$C APE; Cambridge Isotopes) was started and lasted for 4 h. Syringes were weighted before and after the study to determine the exact amount of tracers that were given to infants. The tracer infusion day is depicted in Figure 1.

### Sample collection and analysis

Breath samples were obtained by using the direct nasopharyngeal sampling method described by van der Schoor et al (18). Briefly, a 6F gastric tube (6 CH Argyle; Sherwood Medical) was placed 1–1.5 cm into the nasopharynx, and the end-tidal breath was obtained at 15-min intervals during an isotopic plateau of $[^{13}C]$ bicarbonate between 105 and 180 min. Seven duplicate breath samples were obtained every 10 min during an isotopic plateau of L-[1-$^{13}$C]phenylalanine between 360 and 420 min (Figure 1). $^{13}$C isotopic enrichment in breath samples was analyzed by using an infrared isotope analysis technique (Helifan; Analytic Fischer Instruments) (19). The $^{13}$C enrichment was expressed as the APE above baseline.

### Calculations

The isotopic steady state was represented by a plateau in $^{13}$CO$_2$. Plateaus were determined by using visual inspection and were confirmed by using regression analysis as a slope not significantly different from zero.

The estimated body CO$_2$ production rate (mmol·kg$^{-1}$·h$^{-1}$) was calculated as described previously (14, 17). The fraction of $^{13}$CO$_2$ recovery from L-[1-$^{13}$C]phenylalanine oxidation ($F^{13}$CO$_2$) as a percentage was calculated by using the following equation (20):

$$F^{13}CO_2 = (IE_{PHE} \times i_B) / (i_{PHE} \times IE_B) \times 100\%$$

where $IE_{PHE}$ is the $^{13}$C isotopic enrichment in expired air during [L-$^{13}$C]phenylalanine infusion (APE), $i_B$ is the isotopic enrichment in expired air during L-[1-$^{13}$C]phenylalanine [L-$^{13}$C]bicarbonate (APE), $IE_B$ is the $^{13}$C enrichment in expired air during [13C]bicarbonate infusion.

Phenylalanine flux was not obtained. As shown in our previous study, the test amino acid intake has no effect on the phenylalanine flux (14).

### Statistical analysis

Descriptive data are expressed as means ± SDs. Determination of the methionine requirement (ie, the breakpoint) was performed by using a biphasic linear regression crossover model (21). With the biphasic linear regression analysis, the regression equation was split into 2 parts. For the first part, an intercept and slope were estimated, whereas for the second part, the slope was restricted to zero. Therefore, the estimated intercept of the second line was equal to the breakpoint. The model with the best fit on the basis of the highest $r^2$ was selected. The 95% CIs were calculated. $P < 0.05$ was considered significant. Analyses were performed with STATA software (version 11; StatataCorp LP).

The power analysis could not be performed. We aimed to study 20–35 infants, which was greater than the number of infants used in studies in parenterally fed infants by using the same approach with an intravenous administration of the tracer (22–24).

### RESULTS

#### Subject characteristics

Thirty-three term neonates participated in the study. The neonates were studied at a methionine intake that ranged between 3 and 59 mg·kg$^{-1}$·d$^{-1}$. Subject characteristics are summarized in Table 2. All subjects were growing well before entering the study. The mean (±SD) weight gain rate 3 d before the study was $13 \pm 5$ g·kg$^{-1}$·d$^{-1}$. The mean (±SD) energy intake was $109 \pm 1$ kcal·kg$^{-1}$·d$^{-1}$. The nitrogen intake was equivalent to a protein intake of $3.0 \pm 0.1$ g·kg$^{-1}$·d$^{-1}$. Infants were
clinically stable and considered healthy because they were discharged on the study day or the day after. Primary reasons for admissions were unconjugated hyperbilirubinemia ($n = 15$), pneumonia with a negative blood culture ($n = 6$), asphyxia ($n = 4$), infection suspicion with a negative blood culture ($n = 5$), wet lung ($n = 1$), observation that was due to uterine bleeding ($n = 1$), and pending results of toxoplasmosis, other agents, rubella, cytomegalovirus, and herpes simplex virus ($n = 1$), which were negative. Intravenous antibiotics (penicillins and/or cephalosporins) were given to 28 of the 33 infants.

**TABLE 2**
Subject characteristics and protein and energy intakes before the study of infants who participated in the study ($n = 33$)

<table>
<thead>
<tr>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (kg)</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
</tr>
<tr>
<td>Age on study day (d)</td>
</tr>
<tr>
<td>Weight on study day (kg)</td>
</tr>
<tr>
<td>Weight gain before study (g·kg$^{-1}$·d$^{-1}$)</td>
</tr>
<tr>
<td>Sex (F:M)</td>
</tr>
<tr>
<td>Protein intake before the study (g·kg$^{-1}$·d$^{-1}$)</td>
</tr>
<tr>
<td>Energy intake before the study (kcal·kg$^{-1}$·d$^{-1}$)</td>
</tr>
</tbody>
</table>

All values are means ± SDs.

$^{13}$CO$_2$ enrichments during $[^{13}$C]bicarbonate infusion

The baseline $^{13}$CO$_2$ enrichment was $-$17.04 ± 0.94 Pee Dee Belemnite. The mean $^{13}$CO$_2$ enrichment at isotopic plateau during $[^{13}$C]bicarbonate infusion was 0.0380 ± 0.0032 APE. The corresponding mean CO$_2$ production rate was 23.44 ± 2.04 mmol·kg$^{-1}$·h$^{-1}$. The mean $^{13}$CO$_2$ enrichment at the isotopic plateau and their corresponding CO$_2$ production rate of each infant were plotted against the methionine intake (Figure 2).

$^{1}$-[1-$^{13}$C]phenylalanine oxidation

The mean $^{13}$CO$_2$ enrichment at isotopic plateau during $[1-$[1-$^{13}$C]phenylalanine infusion was 0.0198 ± 0.0024 APE. These $^{13}$CO$_2$-enrichment values and the F$^{13}$CO$_2$ were plotted against

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**FIGURE 2.** A: Mean $^{13}$CO$_2$ enrichment at the isotopic plateau during enteral $[^{13}$C]bicarbonate infusion of each infant plotted against the methionine intake ($n = 33$). B: CO$_2$ production rate of each infant plotted against the methionine intake ($n = 33$). APE, atom percentage excess.
methionine intakes, as shown in Figure 3. As the methionine intake increased, F\(^{13}\)CO\(_2\) decreased. This negative correlation was shown between F\(^{13}\)CO\(_2\) and methionine intakes. Additional increases in methionine intake did not affect the F\(^{13}\)CO\(_2\). With the use of a biphasic linear regression crossover model, the mean methionine requirement was determined to be 38 mg·kg\(^{-1}\)·d\(^{-1}\) (P < 0.0001, \(r^2 = 0.59\)). The upper CI was 48 mg·kg\(^{-1}\)·d\(^{-1}\), and the lower CI was 27 mg·kg\(^{-1}\)·d\(^{-1}\).

**DISCUSSION**

The minimal obligatory methionine requirement of enterally fed term infants was estimated to be 38 mg·kg\(^{-1}\)·d\(^{-1}\) by using the IAAO method. This value is comparable with the estimates of 32–49 mg·kg\(^{-1}\)·d\(^{-1}\) determined by Snyderman et al (8). In the study of Snyderman et al (8), the methionine requirement was determined in 7 infants with postnatal ages between 2 wk and 2 mo by using weight-gain rates and nitrogen retention.

The study diet used by Snyderman et al (8) was an elemental diet that provided a cysteine intake of 64 mg·kg\(^{-1}\)·d\(^{-1}\). Fomon et al (9–11) reported a series of studies with soy-protein formulas with or without a methionine supplement fed to infants over a period of several months. Eight to 13 infants were included in each study diet. The adequacy of the diet and, thus, the adequacy of sulfur amino acid intakes were estimated by measurement of growth, serum chemical indexes, and nitrogen retention. Fomon et al (9–11) concluded that for female infants, a diet with a methionine content of 35 mg/100 kcal was considered adequate; however, a methionine intake of 39 mg/100 kcal failed to meet the requirement for male infants 56 d old.

Although our study was not designed to detect sex differences in the methionine requirement, the average requirement estimates by Fomon et al (9–11) were consistent with our results. Limitations of previous studies were the relative small numbers of subjects studied and the methods used. Growth rates and
nitrogen balance might not be the most sensitive and accurate methods for estimating amino acid requirements.

Courtney-Martin et al (22) determined the methionine requirement in parenterally fed postsurgical neonates by using the IAAO method to be 49 mg · kg\(^{-1}\) · d\(^{-1}\). The diet was devoid of cysteine. To compare their estimates with ours, the route of nutrition intake and the sparing effect of cysteine on the methionine requirement need to be taken into account. Experiments with human fetal tissues showed the lack of activity of cystathionase (25, 26), which is the enzyme involved in the final step in the cysteine-synthesis pathway. However, clinical studies showed the capability of the transsulfuration of methionine to cysteine in term and preterm neonates (27–29) and, thereby, showed the capability of the transsulfuration of methionine to cysteine. To compare their estimates with ours, the route of nutrition intake and the sparing effect of cysteine on the methionine requirement need to be taken into account. Experiments with human fetal tissues showed the lack of activity of cystathionase (25, 26), which is the enzyme involved in the final step in the cysteine-synthesis pathway. However, clinical studies showed the capability of the transsulfuration of methionine to cysteine in term and preterm neonates (27–29) and, thereby, showed the capability of the transsulfuration of methionine to cysteine. To compare their estimates with ours, the route of nutrition intake and the sparing effect of cysteine on the methionine requirement need to be taken into account. Experiments with human fetal tissues showed the lack of activity of cystathionase (25, 26), which is the enzyme involved in the final step in the cysteine-synthesis pathway. However, clinical studies showed the capability of the transsulfuration of methionine to cysteine in term and preterm neonates (27–29) and, thereby, showed the capability of the transsulfuration of methionine to cysteine.

In conclusion, the minimal obligatory methionine requirement is determined to be 38 mg · kg\(^{-1}\) · d\(^{-1}\) for term neonates fed an amino acid based formula provided with an excess of cysteine. Current infant formulas provide excess methionine (49–80 mg · kg\(^{-1}\) · d\(^{-1}\)) when 150 mL · kg\(^{-1}\) · d\(^{-1}\) is consumed (47). The results of our current study provide more scientific knowledge of amino acid needs of infants fed an infant formula, which is necessary to improve infant nutrition.


