

Total sulfur amino acid requirement and metabolism in parenterally fed postsurgical human neonates^{1–3}

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ABSTRACT

Background: Except for tyrosine, the amino acid requirements of human neonates receiving parenteral nutrition (PN) have not been experimentally derived.

Objectives: The objectives were to determine the total sulfur amino acid (TSAA) requirement (methionine in the absence of cysteine) of postsurgical, PN-fed human neonates by using the indicator amino acid oxidation (IAAO) technique with L-[1-¹³C]phenylalanine as the indicator.

Design: Fifteen postsurgical neonates were randomly assigned to receive 1 of 18 methionine intakes ranging from 10 to 120 mg · kg⁻¹ · d⁻¹, delivered in a customized, cysteine-free amino acid solution. Breath and urine samples were collected for the measurement of ¹³CO₂ and amino acid enrichment. Blood samples were collected at baseline and after the test methionine infusion for the measurement of plasma methionine, homocysteine, cystathionine, and cysteine concentrations.

Results: Breakpoint analysis determined the mean TSAA requirements to be 47.4 (95% CI: 38.7, 56.1) and 49.0 (95% CI: 39.9, 58.0) mg · kg⁻¹ · d⁻¹ with the use of oxidation and F¹³CO₂, respectively.

Conclusions: This is the first study to report the TSAA requirement of postsurgical, PN-fed human neonates. The estimated methionine requirement expressed as a proportion of the methionine content of current commercial pediatric PN solutions was 90% (range: 48–90%) of that found in the lowest methionine-containing PN solution. *Am J Clin Nutr* 2008;88:115–24.

INTRODUCTION

Except for tyrosine, the amino acid requirements for parenteral feeding have not been experimentally determined in humans. Instead, the amino acid profile of current commercial amino acid solutions are patterned after reference proteins consumed enterally (1, 2) or on the amino acid concentrations of plasma or cord blood (3, 4).

Our laboratory developed a neonatal piglet model (5) to study amino acid requirements, which was later validated in human neonates for the determination of the tyrosine requirement during parenteral nutrition (PN) feeding (6). Results from the piglet studies have shown that the requirements for several amino acids are lower during parenteral feeding than during enteral feeding (7–9). This suggests that the amino acid profiles of some currently available commercial amino acid solutions are potentially too high. In addition, existing evidence suggests that the inadequate amino acid profile of current PN solutions contributes to the liver cholestasis observed in neonates on long-term PN feeding (10–12).

Methionine is an indispensable sulfur amino acid that donates its sulfur atom to form cysteine during the process of transsulfuration (13). Methionine is also the most important methyl donor in vivo and a precursor for DNA and RNA synthesis (14). However, methionine has been shown to be the most toxic of all amino acids (15, 16), producing cholestatic changes in the liver of animals similar to that observed in human neonates receiving PN feeding (17). Commercial amino acid solutions have a high methionine content in an effort to provide cysteine via the transsulfuration pathway because of the instability of cysteine in solution. This practice, although resulting in high plasma methionine, has not served to normalize the plasma cysteine concentration of PN-fed neonates, which appears low on the basis of current data (18–20). In addition, data from our piglet studies show that the methionine requirement of the PN-fed neonate is 30% less than the enteral requirement (7).

The goals of this study were to determine the total sulfur amino acid (TSAA) requirement (methionine in the absence of cysteine) of PN-fed human neonates by using the indicator amino acid oxidation technique (IAAO) and to measure the plasma amino acid concentrations of several amino acids involved in sulfur amino acid metabolism in response to feeding graded intakes of methionine. On the basis of the results from our piglet model, we predicted that the TSAA requirement of PN-fed humans would be ≈52 mg · kg⁻¹ · d⁻¹.

SUBJECTS AND METHODS

Subjects

Fifteen neonates treated from July 2005 to December 2006 at the Neonatal Intensive Care Unit (NICU), at The Hospital for

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² Supported by grant number FRN 12928 from the Canadian Institutes for Health Research. GCM was supported by scholarships from The Clinician Scientist Training Program, The Hospital for Sick Children, and The Strategic Training Institute in Health Research, The Canadian Institute of Health Research.

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Received October 6, 2007.

Accepted for publication March 14, 2008.

Sick Children, Toronto, Canada were enrolled in this study. The following inclusion criteria were used to determine eligibility: born at ≥ 34 wk gestation and ≤ 28 d chronological age at the time of the study, birth weight and length appropriate for gestational age, medically stable as determined by normal blood test results and lack of a fever or infection, at least 3 d postoperative, and receiving PN providing adequate protein and calories as determined by attending physicians and dietitians. Exclusion criteria included use of mechanical ventilation, small-for-gestational-age status, presence of disease or use of medications known to affect protein and amino acid metabolism, documented infection, fever, unstable medical condition, and receipt of enteral feeding providing $>10\%$ of protein intake.

Neonates were studied ≥ 3 d after surgery for a number of reasons:

- 1) Jones et al (21) showed that the increase in resting energy expenditure (REE) experienced by postsurgical neonates peaks 2 to 4 h after surgery and returns to baseline by 12 to 24 h after surgery. They also showed that substrate utilization was not altered by surgery. Although they showed that the increase in REE was greater in infants undergoing major surgery, REE returned to baseline levels by 24 h.
- 2) Zlotkin et al (22) showed that protein and energy intakes of 2.7 to 3.5 g/kg and of 81 kcal/kg, respectively, resulted in nitrogen retention and growth rates similar to in utero values in premature infants when studied 4 d after surgery.
- 3) We previously reported amino nitrogen flux rates in postsurgical infants (1–4 d after surgery) (23) receiving total PN (TPN) that were similar to those observed in a similar group of infants receiving TPN who had not undergone surgery (24). On the basis of this evidence, it is suggested that protein metabolism does not differ between neonates who have not undergone surgery and postsurgical neonates 3 days after surgery.

Ethical approval for the study was obtained from The Research Ethics Board at The Hospital for Sick Children. Permission was obtained from the attending physician before approaching parents, and written informed consent was obtained from at least one parent before the subjects were enrolled in the study. Study characteristics of the neonates included in the study are presented in **Table 1**.

Experimental design

The IAAO technique (25) was used to determine the TSAA requirement. The IAAO technique is based on the observation that the partitioning of any indispensable amino acid between oxidation and protein synthesis is sensitive to the level of the most limiting amino acid in the diet (26). Because amino acids are not stored in the body, when an indispensable amino acid is limiting in the diet for protein synthesis, all other amino acids are in excess and are therefore oxidized. Therefore, increasing the level of the limiting amino acid in graded amounts from deficient to excess will also increase the uptake of all dietary amino acids for protein synthesis. As their uptake for protein synthesis is increased, the portion of the indicator that is oxidized will decrease until the requirement of the test amino acid (in this case methionine) is reached. Once the requirement of the test amino acid is reached, further increase in its intake will have no further effect on the indicator amino acid. The first point after which an

TABLE 1

Subject characteristics of parenterally fed neonates who received various methionine intakes and no cysteine during the determination of the total sulfur amino acid requirement¹

	Value (n = 15)
Birth weight (kg)	2.7 \pm 0.5 ²
Birth length (cm)	47.5 \pm 25
Head circumference (cm)	32.6 \pm 1.7
Gestational age (wk)	37 \pm 2
Postnatal age (wk)	1.8 \pm 0.6
Postconceptional age (wk)	39 \pm 2
Sex (F:M)	4:11
Study weight (kg)	2.8 \pm 0.5

¹ Diagnoses: esophageal atresia/tracheoesophageal fistula (n = 20), jejunal atresias (n = 1), duodenal web (n = 1), gastroschisis (n = 5), multiple small bowel atresias (n = 1), duodenal atresia (n = 1), meconium plug syndrome (n = 1), omphalocele (n = 1).

² $\bar{x} \pm$ SD (all such values).

increase in the test amino acid has no further effect on the oxidation of the indicator is the breakpoint, or the mean requirement—the point at which the test amino acid is no longer limiting for protein synthesis. The inverse relation between phenylalanine oxidation and protein synthesis is supported by the experiment of Ball and Bayley (26), in which L-[1-¹⁴C]phenylalanine was used as an indicator of the protein requirement in young pigs. The results show that the recovery of radioactivity in the breath was inversely related to the recovery of radioactivity in liver tissue when dietary protein was increased from deficient to adequate, which demonstrated that the oxidation of the indicator was inversely related to protein synthesis. In addition, we showed in humans that the requirement for tyrosine determined by the IAAO method using lysine as an indicator was similar to that determined by measuring the hydroxylation of phenylalanine to tyrosine in apolipoprotein B-100 (27).

Blood samples were drawn from each subject for the measurement of amino acid concentrations. Each study lasted for a total of 48 h. During the first 24 h, each subject received a commercial amino acid solution (Primene; Baxter Laboratories, Mississauga, Canada) (**Table 2**), dextrose, and a 20% lipid solution (Intralipid; Fresenius Kabi, Uppsala, Sweden) to ensure an adequate protein and nonprotein energy intake. Standard amounts of vitamin and minerals were provided in the form of a liquid supplement (Multi-12/K1; providing a mixture of fat and water-soluble vitamins) formulated for use in intravenous feeding. All vitamins and minerals met current Dietary Reference Intake recommendations. The mean (\pm SD) sulfur amino acid intake of subjects during the first 24 h (day 1) was 52.97 \pm 9.04 and 69.42 \pm 4.17 mg \cdot kg⁻¹ \cdot d⁻¹ for cysteine and methionine, respectively, for a total intake of 122.39 \pm 8.93 mg \cdot kg⁻¹ \cdot d⁻¹ TSAA. The base amino acid solution indicated above was chosen for a number of reasons, including because it is the amino acid used in the NICU at our hospital (The Hospital for Sick Children, Toronto) and has been the amino acid solution in use for >10 y, and it is also used in NICUs in Europe (28, 29). When the plasma amino acid concentration of neonates receiving the amino acid solution used in this study was compared with that of breastfed infants (30), most of the amino acids were within the reference range; only 2 indispensable amino acids, valine and lysine, were higher and

TABLE 2

Amino acid concentration of parenteral solutions administered to neonates on day 1 (adaptation day) and day 2 (study day)

Amino acid	Day 1	Day 2
	<i>g/100 g</i>	
Isoleucine	6.7	6.7
Leucine	9.9	9.9
Valine	7.6	7.6
Lysine (lysine-HCl)	10.9	13.62
Methionine	2.4	Variable ¹
Cysteine	1.9	0
Phenylalanine	4.2	3.7
Tyrosine	0.9	0
Glycyl-tyrosine	0	5.25
Threonine	3.7	3.7
Tryptophan	2.0	2.0
Histidine	3.8	3.8
Arginine	8.4	9.66
Glycine (total)	4.0	4.0
Alanine	7.9	7.9 ²
Aspartate	6.0	5.0
Glutamate	9.9	9.9
Proline	3.0	3.0
Serine	4.0	4.0
Taurine	0.6	0.6
Ornithine	2.2	0
Total amino acids	100.00	94.15 ³

¹ Value based on test intake level.

² Plus additional amounts to make the solution isonitrogenous.

³ Solution on day 2 represented 94.15% of the total protein/total amino acid. Phenylalanine was provided in the parenteral solution (1.9 g/100 g) and as isotope (1.8 g/100 g).

tyrosine was lower (29). This low tyrosine concentration suggests that tyrosine is limiting in the amino acid solution used, an observation that was confirmed by our group (3). Subsequently, we determined the tyrosine requirement of TPN-fed human neonates (6), which formed the basis for the amount of tyrosine used in the solutions made for this study. We used glycyl-tyrosine as our tyrosine source instead of *N*-acetyl-tyrosine, because glycyl-tyrosine has been found to be an available source of tyrosine for neonates (6), unlike *N*-acetyl-tyrosine (4, 31). Additionally, we used data from our piglet work to make adjustments to the arginine content of the amino acid base solution. Our piglet data suggest that the arginine requirement is greater with intravenous than with enteral feeding (32).

During the second 24-h period, subjects were randomly assigned to receive 1 of 18 methionine intakes ranging from 10 to 120 mg · kg⁻¹ · d⁻¹ and no cysteine. Hence, a separate amino acid solution was prepared for this part of the study (Table 2). To control the methionine intake, both methionine and cysteine were removed from this solution. Methionine was added back on the study day in an amount consistent with the intake being studied on that day. Again, grams of fat and protein and total calories delivered were determined by the attending physician and dietitians. Each subject was intravenously fed via a central line and received a fluid intake between 140 and 160 mL · kg⁻¹ · d⁻¹.

Study diets

The base amino acid profile of the amino acid solutions used on day 1 (adaptation period) and day 2 (test period) is presented

in Table 2. The amino acid profile of the base amino acid solution used was based on the amino acid composition of cord blood. The amino acid solution used on day 2 (test period) was prepared using human parenteral grade amino acids (Ajinomoto Company Inc, Japan, via LV Lomas, Brampton, Canada) in our research laboratory under sterile conditions. The profile of the base amino acid was followed, with some modifications; methionine was varied to meet the level of the test intake for each neonate, cysteine was removed, and tyrosine was provided in excess as the dipeptide glycyl-tyrosine at a level of 4 g/100g (6). The excess tyrosine was provided to facilitate the channeling toward oxidation of any tyrosine synthesized from phenylalanine (33), thereby making phenylalanine a more sensitive indicator. Phenylalanine was provided at a total amount of 3.7 g/100 g, and the amount used for tracer infusion was subtracted from the base solution. Arginine was increased from 8.4 to 9.66 g/100 g (32, 34), and aspartate was decreased from 6.0 to 5.0 g/100g to accommodate for the increased amount of nitrogen provided by arginine. Alanine was used to balance the nitrogen and make the solution isonitrogenous. Methionine and alanine were prepared as separate solutions in concentrations of 20 and 50 mg/mL, respectively. All prepared solutions were filter sterilized in the Research Pharmacy at The Hospital for Sick Children by being passed through a 0.22- μ m filter. Solutions were subsequently shown to be sterile and free of bacterial growth over 7 d in culture and to be proven pyrogen-free by the limulus amoebocyte lysate test (35). The chemical composition of the solutions was verified by amino acid analysis with the use of HPLC and an analysis of total nitrogen.

On each study day, vitamins and minerals were added to the solutions before delivery to the infants. All vitamins were supplied in a commercial solution (Multi-12K1; Baxter Corporation), which provides a combination of fat and water-soluble vitamins, formulated for use in pediatric parenteral solutions. Cofactors involved in methionine metabolism, vitamin B-12, vitamin B-6, and folic acid were provided in the Multi-12K1 solution at 1.2 to 1.5 times the requirement (36). The mineral solution provided calcium, phosphorus, magnesium, zinc, copper, manganese, iodine, chromium, and selenium. Nutrient intakes for each individual neonate were prescribed by the attending physician and dietitian. All subjects were receiving adequate protein and calories (22, 37). Nonprotein calories were provided as dextrose and a 20% lipid solution (Table 3).

The study began with each neonate receiving the base TPN/lipid solution for a total of 24 h to ensure that all neonates started the test infusion with similar amino acid profiles. For the second 24-h period, neonates received the test solution containing a randomly assigned methionine intake from 10 to 120 mg · kg⁻¹ · d⁻¹ and no cysteine. The test level of methionine was added to the test solution on the study day. All neonates received the test TPN solution until the end of the study, at which time the TPN solution that they had been receiving before the study was resumed.

Baseline blood work performed for clinical monitoring was reviewed for each subject before the study began. All subjects had normal sodium, potassium, calcium, phosphorus, and pH. Five subjects had high chloride concentrations, between 107 and 113 mmol/L (normal range: 96–106 mmol/L). This was corrected by replacing the chloride with acetate in the TPN solution.

TABLE 3

Methionine, amino acids, lipids, carbohydrates, and total energy intakes on study day (day 2)

Subject no. and study	Methionine	Amino acids	Lipids	Carbohydrates	Energy
	$\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$	$\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$	$\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$	$\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$	$\text{kJ} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$
1, 1	118.7	2.86	2.98	14.44	346.94
2, 2	9.5	2.64	2.96	13.89	355.3
3, 3	99.86	2.91	2.94	11.65	338.58
3, 4	29.95	2.91	2.94	11.65	334.4
4, 5	31.8	2.7	2.98	13.4	380.38
6, 6	49.14	2.7	3.3	13.69	372.02
7, 7	77.6	2.83	2.88	14.56	367.84
7, 8	14.87	2.84	2.88	14.32	367.84
8, 9	39.1	2.98	3.0	13.9	338.58
9, 10	19.16	2.93	3.0	13.9	363.66
10, 11	55.6	2.71	3.0	13.38	367.84
11, 12	61.51	2.91	2.45	14.2	353.3
12, 13	107	2.8	3.15	11.52	334.4
13, 14	86.3	3.36	2.9	14.05	372.02
13, 15	61.5	3.1	2.98	14.05	351.12
14, 16	33.9	3.1	3.3	13.78	380.38
15, 17	24.5	2.84	2.93	12.84	351.12
15, 18	43.7	2.9	3.07	11.87	338.58
$\bar{x} \pm \text{SD}$		2.89 ± 0.17	2.98 ± 0.18	13.39 ± 1.03	356.46 ± 15.63

Tracer protocol

The tracer protocol was started at the beginning of the second 24-h period, at the time that the test bag of PN was hung. Phenylalanine was used as the indicator amino acid with L-[1-¹³C]phenylalanine, [99 atom percent excess (APE) (Cambridge Isotope Laboratories, Woburn, MA) as the isotope for the measurement of phenylalanine kinetics. An intravenous priming dose of L-[1-¹³C]phenylalanine was given at $15.6 \mu\text{mol}/\text{kg}^{-1}$ over 15 min followed by a continuous intravenous infusion of $13 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ for the remainder of the 23.75 h. The amount of L-[1-¹³C]phenylalanine given was subtracted from the phenylalanine provided in the PN solution to maintain a total phenylalanine intake of $111 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ($3.7 \text{ g}/100 \text{ g}$). Tyrosine was provided at $120 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ to ensure an excess of tyrosine (38, 39). L-[1-¹³C]Phenylalanine was analyzed by liquid chromatography mass spectrometry/mass spectrometry (LCMS/MS) using a chiral column for the presence of D-[1-¹³C]phenylalanine. There was no detectable D-[1-¹³C]phenylalanine in the tracer.

The infusion time of the tracer was extended to 23.75 h for practical reasons. Given our goal to make these studies as least invasive as possible, an extended tracer infusion time was necessary to allow enough time for urine collection because babies do not void on demand. We had previously found that we needed ≥ 3 h to get a urine sample. Although some infants in our study had lots of urine, it took up to 4 h to get the required sample from other infants.

The phenylalanine solutions were prepared in the Manufacturing Pharmacy at the Hospital for Sick Children and were sterilized and tested for bacteria and pyrogens as described above. The solutions were kept at 4°C until used.

Sample collection and analysis

Urine samples were collected from cotton pads placed on the inside of the diapers for the measurement of baseline and steady state tracer amino acid enrichment. Three baseline urine samples

were collected before the start of the intravenous isotope infusion. Three to 5 samples were collected beginning 12 h after the start of the isotope infusion until the end. Urine was stored at -20°C until analyzed.

Expired carbon dioxide was collected at baseline and at isotopic steady state from a ventilated hood system using a portable carbon dioxide analyzer (1400 series; Servomex, Westech Industrial Ltd, Mississauga, Canada) and mass flow meter (5860 series; Brooks, Trillium Measurement and Control, Stouffville, Canada). Briefly, a clear plastic hood was placed over the infant's head through which the baby had access to room air. The carbon dioxide concentration in the hood was monitored, and expired carbon dioxide was sampled when the concentration was between 0.2% and 0.5%. To minimize the variability in the ratio of ¹²CO₂ to ¹³CO₂, we kept the carbon dioxide concentration within the hood between 0.35% and 0.45% during the measurements. Three baseline samples were collected before the start of the intravenous isotope infusion, and 5 samples were collected at 2-h intervals, starting from 12 h after the start of the isotope infusion. Each sample was collected over a period of 10 min by bubbling the sampled carbon dioxide into 10 mL NaOH through a reflux condenser to form NaHCO₃. The NaHCO₃ was then injected into Vacutainers (Beckton Dickinson and Company, Franklin Lakes, NJ) and stored at -20°C until analyzed. The carbon dioxide production rate was measured between 18 and 23 h after the start of the intravenous isotope infusion.

Two 0.5-mL samples of blood were collected: one at the end of the first 24-h period before the start of the test PN solution and the other at the end of the second day, when subjects had received the test methionine intake. Blood was analysed for plasma amino acid, homocysteine, and cystathionine concentrations.

The enrichment of L-[1-¹³C]phenylalanine in urine was analyzed using a triple quadrupole mass analyzer (API 4000; Applied Biosystems/MDS SCIEX, Concord, Canada) coupled to an Agilent 1100 HPLC system (Agilent, Mississauga, Canada), as

previously described (40). We used a Chirobiotic T Chiral Column purchased from Sigma-Aldrich to separate the D-[1-¹³C]phenylalanine from L-[1-¹³C]phenylalanine. Isotopic enrichment was expressed as mole percent excess and was calculated from peak area ratios at isotopic steady state at baseline and plateau. The use of urine as a surrogate of plasma enrichment has been used by our group in an effort to make our studies less invasive for vulnerable groups, such as women, children, and neonates. The urine enrichments were found to be highly correlated with the plasma enrichment (41) and gave similar results, except in cases in which the presence of the D-isomer in the isotope infused is in excess of 0.2% or within any detectable range (42).

For the analysis of ¹³C enrichment in breath, samples were prepared by liberating the carbon dioxide from the NaHCO₃ by mixing 200 μL sample (NaHCO₃) with 200 μL H₃PO₄ into an evacuated tube. Enrichment of ¹³C in breath was analyzed by using a continuous-flow isotope ratio mass spectrometer (20/20 isotope analyzer; PDZ Europa Ltd, Cheshire, United Kingdom). Enrichments were expressed as APE compared with a reference standard of compressed carbon dioxide gas.

Plasma was separated from whole blood within 10 min of the blood being drawn from each subject. Plasma amino acid concentrations were analyzed by reversed-phase HPLC using the PICO.TAG (Waters, Milford, MA) precolumn derivitization method with phenylisothiocyanate as the derivative. The amino acid concentrations were determined by using an external standard. Plasma total homocysteine, total cysteine, and cystathionine concentrations were determined by LCMS/MS, using a bench top triple quadrupole mass spectrometer (API 4000; Applied Biosystems/MDS SCIEX) operated in positive ionization mode with the TurboIonSpray ionization probe source (operated at 5.8 kV). This was coupled to an Agilent 1100 HPLC system (43).

Tracer kinetics

The model of amino acid kinetics used in this study is based on the model of Matthews et al (44). Isotopic steady state in the L-[1-¹³C]phenylalanine enrichment in the urine and ¹³CO₂ in breath was represented by unchanging values in each of the data points at baseline and plateau. Phenylalanine flux (in μmol · kg⁻¹ · h⁻¹) was calculated from the dilution of the intravenously administered L-[1-¹³C]phenylalanine into the metabolic pool at isotopic steady state using urinary enrichment of L-[1-¹³C]phenylalanine as a representation of plasma enrichment (41). The rate of phenylalanine oxidation, represented by the rate of appearance of ¹³CO₂ in breath (F¹³CO₂), in μmol · kg⁻¹ · h⁻¹, was calculated according to the model of Matthews et al (44). A correction factor of 0.82 was used to account for the retention of ¹³CO₂ in the body's bicarbonate pool in the fed state (45). This individual correction factor was used instead of the algorithm because we calculated the F¹³CO₂ using the algorithm and the set correction factor of 0.82 and found the results to be the same. Because the range in energy intakes was narrow, we judged it simpler if we presented our data using a constant correction factor. Phenylalanine oxidation (in μmol · kg⁻¹ · h⁻¹) was calculated from the F¹³CO₂ data and the urinary L-[1-¹³C]phenylalanine enrichment (44).

Statistical analysis

The first and second level of methionine intake studied were the highest and lowest intakes as a means of bracketing the predicted breakpoint to determine whether the correct range of intakes was chosen. Subsequent methionine intakes were completely randomized, with methionine serving as the main treatment effect. The effect of methionine intake on phenylalanine flux, oxidation, and F¹³CO₂ was tested by using analysis of variance (ANOVA) with the PROC GLM procedure (SAS version 9.1; SAS Institute Inc, Cary, NC).

Determination of the mean methionine requirement (breakpoint) was performed using a 2-phase linear regression crossover model (46), as previously described (47). Regression analysis variables were dietary methionine intake as the independent variable and F¹³CO₂ and phenylalanine oxidation as the dependent variables. Selection of the best model was determined by factors relating to fit (significance of the model and *r*²) and estimate of variation about the model (CV and SE of the estimate). The safe population intake was estimated by determining the upper 95% confidence limits of the breakpoint estimate (46).

The effect of methionine intake on plasma amino acid concentration was tested by using ANOVA. Because the baseline plasma amino acid concentrations were variable and the infants did not each receive the same protein intake as was planned, we chose to use the difference in amino acid concentration between baseline and after subjects received the test methionine intake as the main dependent variable. Independent variables tested were age, subject, and methionine intake. All statistical analyses were performed by SAS version 9.1 for WINDOWS (SAS Institute Inc, Cary, NC). Statistical significance was established at *P* ≤ 0.05.

RESULTS

Clinical characteristics and nutrient intakes

Clinical characteristics and diagnoses for the 15 subjects studied are presented in Table 1. Four of the 15 infants were above birth weight, 10 infants had just regained birth weight, and 1 infant, who was 1-wk-old, had not yet regained birth weight. Three subjects received 2 intake levels of methionine for a total of 18 indicator oxidation studies in 15 subjects. Because PN was prescribed by the attending physician and dietitian, the exact nutrient intakes were dependent on the total volume of parenteral nutrition infused. The average energy and protein intakes were 356.46 ± 15.63 kJ · kg⁻¹ · d⁻¹ (85 ± 3.74 kcal · kg⁻¹ · d⁻¹) and 2.89 ± 0.17 g · kg⁻¹ · d⁻¹, respectively (Table 3). The mean (±SD) grams of lipids and carbohydrate provided were 2.98 ± 0.18 and 13.39 ± 1.03, respectively (Table 3).

Urinary amino acid and expired CO₂ enrichment

Isotopic steady state (plateau) was achieved for all neonates by 12 h after the start of the isotope infusion and was defined by the absence of a significant slope between the data points at plateau. The variation in urinary L-[1-¹³C]phenylalanine at plateau was <10%, whereas the variation in expired ¹³CO₂ enrichment within the plateau was <1%.

Phenylalanine kinetics

The mean (±SD) phenylalanine flux of these TPN-fed human neonates was 108.7 ± 9.8 μmol · kg⁻¹ · h⁻¹. By ANOVA, there

was no significant relation between methionine intake and phenylalanine flux ($P = 0.34$). This lack of change in flux in response to changes in methionine intake indicates that differences observed in phenylalanine oxidation and $F^{13}CO_2$ in response to methionine intake were related to a partitioning of amino acids between oxidation and protein synthesis. One of the strengths and key criterion of the IAAO method is that the flux of the indicator amino acid (phenylalanine in this study) does not change in response to feeding graded intakes of the test amino acid, in this case methionine. The flux of the indicator amino acid remains constant because the total intake remains constant at all levels of intake of the test amino acid. We kept the phenylalanine intake exactly the same at all methionine intakes by deducting the amount of the tracer from the total dietary intake and making up the difference in the TPN solution. Because the intake of the indicator amino acid remains constant at all levels of intake of the test amino acid, the pool size does not change. This is an advantage over the direct amino acid oxidation (DAAO) method, in which the free amino acid pool size of the test amino acid changes with each increment in the test amino acid, which results in variable dilution of the tracer within the pool and thus increases the variation and decreases the sensitivity of the estimate.

Methionine intake had a significant effect on both phenylalanine oxidation and $F^{13}CO_2$ ($P < 0.001$). As the methionine intake increased from 9.5 to 47.4 $mg \cdot kg^{-1} \cdot d^{-1}$, phenylalanine oxidation decreased ($P < 0.001$); further increases in methionine intake did not affect phenylalanine oxidation (Figure 1). Similarly, there was a decrease in $F^{13}CO_2$ in response to methionine intake to the level of 49.0 $mg \cdot kg^{-1} \cdot d^{-1}$ methionine, after which a further increase in methionine intake produced no change in $F^{13}CO_2$ (Figure 2). The decreases in phenylalanine oxidation and $F^{13}CO_2$ until the breakpoint were similar for both endpoints used and reflects the sensitivity of the urine measurement when compared with the more direct $F^{13}CO_2$ estimate. The lack of change in phenylalanine oxidation after the breakpoint indicates that, under the conditions of the study, the test amino acid (methionine) was no longer limiting for protein synthesis. It is possible that other amino acids were then limiting for protein synthesis. However, as detailed in Subjects and Methods, the

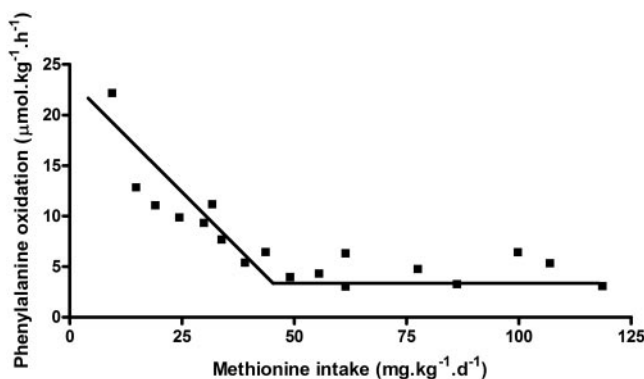


FIGURE 1. Parenteral methionine requirement. The effect of increasing methionine intake on phenylalanine oxidation in total parenteral nutrition-fed human neonates ($n = 18$). Methionine intake had a significant effect on phenylalanine oxidation ($P < 0.001$, $r^2 = 0.81$; ANOVA). A 2-phase linear regression crossover model estimated the breakpoint (mean methionine requirement) to be 47.4 $mg \cdot g^{-1} \cdot d^{-1}$. The safe population intake estimated by determining the upper 95% confidence limits of the breakpoint estimate was 56.1 $mg \cdot g^{-1} \cdot d^{-1}$ with a lower CI of 38.7 $mg \cdot g^{-1} \cdot d^{-1}$.

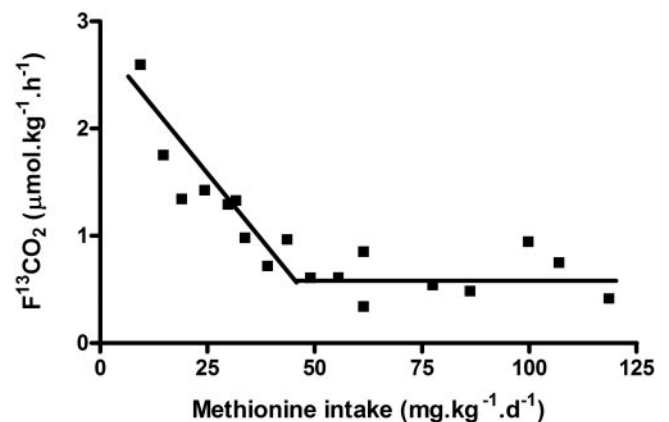


FIGURE 2. Parenteral methionine requirement. The effect of increasing methionine intake on $F^{13}CO_2$ in total parenteral nutrition-fed human neonates ($n = 18$). Methionine intake had a significant effect on phenylalanine $F^{13}CO_2$ ($P < 0.001$, $r^2 = 0.82$; ANOVA). A 2-phase linear regression crossover model estimated the breakpoint (mean methionine requirement) to be 49.0 $mg \cdot g^{-1} \cdot d^{-1}$. The safe population intake estimated by determining the upper 95% confidence limits of the breakpoint estimate was 58.0 $mg \cdot g^{-1} \cdot d^{-1}$ with a lower CI of 39.9 $mg \cdot g^{-1} \cdot d^{-1}$.

amino acid concentrations in the TPN solution used in the current study met or exceeded the concentrations derived from our piglet studies (8, 9, 32, 34) and our prior human neonatal studies (3, 6).

To determine the methionine requirements, the data were partitioned between 2 distinct regression lines (Figures 1 and 2) by using the 2-phase linear regression crossover model. A breakpoint was identified in phenylalanine oxidation at 47.4 $mg \cdot kg^{-1} \cdot d^{-1}$ ($P < 0.001$, $r^2 = 0.81$; Figure 1) and $F^{13}CO_2$ at 49.0 $mg \cdot kg^{-1} \cdot d^{-1}$ ($P < 0.001$, $r^2 = 0.82$; Figure 2). The 95% confidence limits of phenylalanine oxidation and $F^{13}CO_2$ were determined to be 38.7–56.1 and 39.9–58.0 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively (Figures 1 and 2).

Plasma amino acid concentration

Per ANOVA, with the difference between plasma methionine concentrations at baseline, and in response to graded intakes of methionine as the dependant variable, methionine intake had a significant effect on plasma methionine concentration ($P = 0.0439$). The difference in plasma methionine showed a linear response to methionine intake; at lower intakes of methionine, plasma methionine decreased but with graded intakes of methionine, plasma methionine concentration showed a linear increase ($r^2 = 0.277$; Figure 3).

The difference between plasma homocysteine concentration at baseline and in response to graded intakes of methionine was significantly affected by methionine intake ($P < 0.0001$). The plasma homocysteine concentration increased linearly in response to graded intakes of methionine, with 68% of the difference in homocysteine concentration being account for by changes in methionine intake ($r^2 = 0.677$; Figure 4).

There was a significant effect of methionine intake on the difference in plasma cystathionine concentrations between baseline and in response to graded intakes of methionine ($P = 0.0003$). The difference in plasma cystathionine concentrations also showed a linear response to graded intakes of methionine, with 59% of the difference in plasma cystathionine concentration being explained by changes in methionine intake ($r^2 = 0.586$; Figure 5).

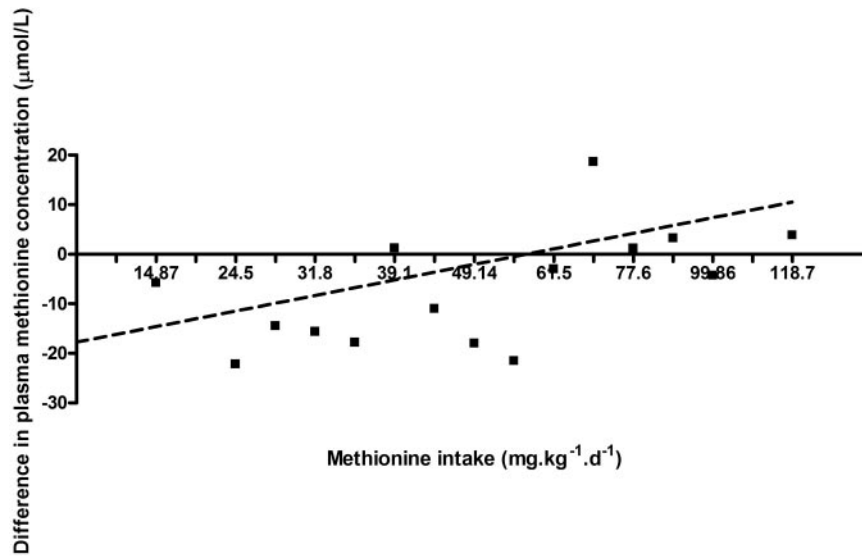


FIGURE 3. Difference between plasma methionine concentrations at baseline and in response to graded intakes of methionine ($n = 15$ because of technical problems with the methionine measurement in 3 studies). Methionine intake had a significant effect on the difference in plasma methionine concentrations ($P = 0.0439$; ANOVA with PROC GLM); 28% of the difference in methionine concentration was explained by methionine intake ($r^2 = 0.277$). The regression equation for the calculation of the difference from baseline in plasma methionine concentration in response to increasing methionine intakes is given by $y = -18.39 + 0.206x$. The 95% CIs of the estimate were -13.43 and -0.65 .

The plasma cysteine concentration significantly decreased from baseline in response to providing the TSAA intake as methionine only ($P < 0.0001$). The decrease in plasma cysteine

concentration ranged from $-51.5 \mu\text{mol/L}$ at the lowest methionine intake to $-141.0 \mu\text{mol/L}$ at the highest methionine intake, with a mean decrease of $-65.5 \mu\text{mol/L}$. However, there was no effect of graded intakes of methionine on plasma cysteine concentrations.

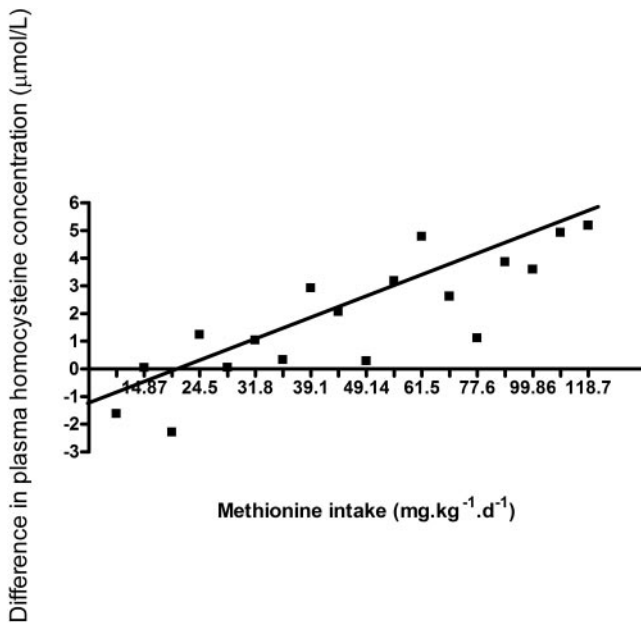


FIGURE 4. Difference between plasma homocysteine concentrations at baseline and in response to graded intakes of methionine ($n = 18$). Methionine intake had a significant effect on the difference in plasma homocysteine concentrations ($P < 0.0001$; ANOVA with the PROC GLM). There was a linear increase in the difference in plasma homocysteine concentration in response to graded intakes of methionine; 68% of the difference in plasma homocysteine concentration was explained by the changes in methionine intake ($r^2 = 0.677$). The regression equation for the calculation of the difference from baseline in plasma homocysteine concentration in response to methionine intake is given by $y = -1.104 + 0.055x$. The 95% CIs of the estimate were 0.76 to 2.94 .

DISCUSSION

This is the first report in which complete parenteral amino acid solutions were prepared in a research laboratory for use in human infants to determine amino acid needs. Our test amino acid solution was based on one particular pediatric solution, the amino acid balance of which differs in part from others on the market (48); hence, our results must be viewed in light of the test conditions used. Our experience in making such solutions for parenterally fed neonatal piglets (7–9) provided the knowledge required to embark on these complex human studies. We chose to study TSAA needs as methionine, because methionine is considered the most toxic of parenteral amino acids (13). We previously showed in piglets that parenteral methionine requirements were 69% of those in enterally fed piglets (7). The TSAA requirement estimated from our piglet study was $0.26 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (7). Piglets were fed $15 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ protein. Because piglets grow at ≈ 5 times the rate of human neonates, we estimated that, at a protein intake of $3 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, the TSAA requirement of PN-fed human neonates would be $\approx 52 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$.

The mean and 95% confidence estimates (Recommended Dietary Allowance) of the TSAA requirement were determined in the current experiment to be 49 and $58 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, respectively. Given the inherent errors of converting piglet estimates to human infants, mean predicted requirement estimates of $52 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (piglet) and $49 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (infant) are considered to be remarkably similar. Because the values obtained for the infant are so close to the values we predicted from the piglet

studies, we believe that this further validates the use of the piglet TPN model to determine the pattern of amino acids needed to optimize protein synthesis and hence growth in human neonates (3, 6, 48).

The lack of change in phenylalanine flux with varying methionine intakes in the current study provides evidence that the differences in oxidation and $F^{13}CO_2$ reflect a shift in the partitioning of amino acids between oxidation and protein synthesis. This indicates that, at a mean methionine intake of $49 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, methionine was no longer limiting for protein synthesis. Indeed, in previous studies in which cysteine was supplemented to cysteine-free TPN, cysteine supplementation did not enhance nitrogen retention in infants (19, 49). In addition, in our piglet studies, in which methionine requirements were determined with and without cysteine (7, 50), a comparison of phenylalanine oxidation between the 2 studies showed no difference in oxidation, which indicated that there was no difference in whole-body protein synthesis with or without dietary cysteine. These data, along with those of others (51, 52), clearly show that cysteine is not a dietary indispensable amino acid in either human neonates or piglets.

The mean plasma methionine concentration of $34.8 \pm 12.5 \text{ } \mu\text{mol/L}$ observed when the infants were adapted to the baseline TPN solution was very similar to that previously reported in breastfed and TPN-fed babies (4, 30, 49). Plasma methionine showed an overall significant decrease in response to graded intakes of methionine, which was mainly due to the decrease observed below the methionine requirement. Once the requirement was reached, the difference in plasma methionine was closer to zero (Figure 3). This pattern of response was similar to that observed by Tontisirin et al (53), when plasma tryptophan was used to determine tryptophan requirements in the elderly.

The increase in plasma homocysteine in response to graded intakes of methionine (Figure 4) was similar to that observed in our piglet study (54). These data and our piglet data (54) clearly show that methionine intake during TPN feeding has a significant positive effect on plasma homocysteine concentrations. Plasma homocysteine is of concern because a mean homocysteine concentration of $>8.5 \text{ } \mu\text{mol/L}$ has been linked to ischemic and hemorrhagic stroke in infants and children (55, 56). Although the mean plasma homocysteine concentration did not reach such high levels in the current study, it is possible that PN solutions with a higher methionine content could result in more undesirable homocysteine concentrations. The provision of the TSAA as a balance of methionine and cysteine is recommended because the provision of a portion of the total sulfur amino acids as cysteine was found to decrease transmethylation of methionine to homocysteine and increase remethylation of homocysteine to methionine (57). More importantly, provision of cysteine reduces the total amount of methionine required in the diet (58).

Plasma cystathionine also responded linearly to graded intakes of methionine; however, the difference in cystathionine concentration from baseline was only positive at the higher intakes of methionine. At methionine intakes $<61.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, the difference in plasma cystathionine was negative (Figure 5). This was surprising because it is generally accepted that the enzyme cystathionase is underdeveloped in human neonates (59, 60), and elevations in plasma cystathionine have been reported in preterm ($<32 \text{ wk}$ gestation) neonates (61). The plasma cystathionine concentration observed in the current study; however, is

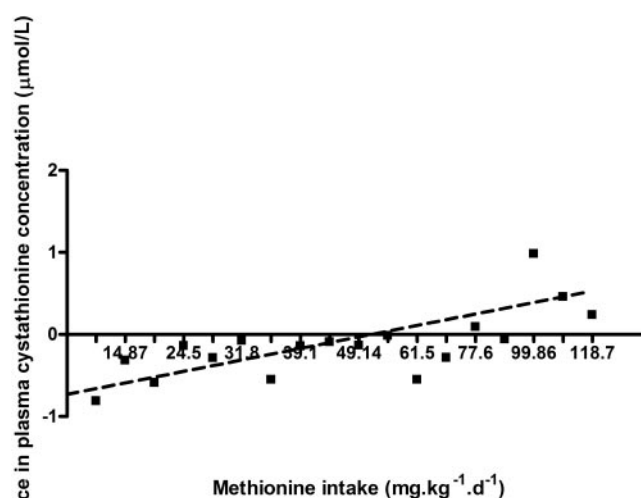


FIGURE 5. Difference between plasma cystathionine concentrations at baseline and in response to graded intakes of methionine ($n = 18$). Methionine intake had a significant effect on the difference in plasma cystathionine concentrations ($P < 0.0003$; ANOVA with the PROC GLM); 59% of the change in plasma cystathionine concentration was explained by changes in methionine intake ($r^2 = 0.586$). The regression equation for the calculation of the difference from baseline in plasma cystathionine concentration in response to methionine intake is given by $y = -0.69 + 0.01x$. The 95% CIs of the estimate were -0.36 to 0.07 .

consistent with that observed in a cohort of >4000 neonates, which ranged from 0.2 to $1.53 \text{ } \mu\text{mol/L}$ (62).

The plasma cysteine concentration decreased significantly from baseline in response to consumption of the test TPN solution devoid of cysteine. The decrease in plasma cysteine, especially at the higher methionine intakes, was likely due in part to the mode of feeding because it has been found that the gastrointestinal tract is a significant site of transsulfuration (63) and that extrahepatic tissues have limited capacity for transsulfuration (64). However, the observed concentrations were higher than those of the term breastfed infants in whom concentrations of $153 \text{ } \mu\text{mol/L}$ were reported (30). Zlotkin et al (49) reported plasma methionine and cysteine concentrations of 105.0 and $59.6 \text{ } \mu\text{mol/L}$ in neonates receiving cysteine-free PN providing 127 mg/kg methionine. The lower cysteine concentrations observed by Zlotkin et al (49) may have been related to the use of the automated amino acid analysis method, which has been shown to underestimate cysteine concentrations in plasma and urine (65).

Although cysteine was not provided in this study, our flux oxidation and $F^{13}CO_2$ data provide evidence that, at a mean intake of $49 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ methionine, protein synthesis was no longer limiting in the subjects and, hence, cysteine availability was most likely adequate. This is supported by evidence that neonates fed cysteine-free TPN are able to maintain adequate growth and nitrogen balance (49, 66).

We concluded that the TSAA (as methionine only) requirement of postsurgical PN-fed human neonates is lower than currently in commercially available PN solutions. We observed that plasma methionine and homocysteine concentrations increase in response to high intakes of methionine. Because methionine produces cholestatic changes in the liver of animals, and a high homocysteine concentration is implicated in stroke in

infants and children, we believe that the current commercial PN solutions need to have a lower SAA content.

We gratefully acknowledge the contributions of all of the infants who participated in this study and their parents for allowing them to do so. We thank all of the nurses and doctors in the NICU at the Hospital for Sick Children for their remarkable teamwork and corporation. We especially thank Nicole de Silva and Felice Pettolino for their help in subject recruitment and study set up and Jacob Langer for his support throughout the study. We are also grateful to the Baxter Corporation (Mississauga, Canada) for supplying us with the first set of amino acids used to perform all initial testing necessary before the start of the study. We gratefully acknowledge the help of the TPN Research Pharmacy at The Hospital for Sick Children, especially Mark Bedford.

The authors' responsibilities were as follows—GC-M: study design, subject recruitment, data collection, sample and data analysis, and manuscript writing; KPC: data collection and manuscript writing; AMM: subject recruitment and study set up; and ROB and PBP: study design, data analysis, and manuscript writing. None of the authors had any conflicts of interest.

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