

# Arginine Is Synthesized From Proline, Not Glutamate, in Enterally Fed Human Preterm Neonates

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**ABSTRACT:** In neonatal mammals, arginine is synthesized in the enterocyte, with either proline or glutamate as the dietary precursor. We have shown several times in piglets that proline is the only precursor to arginine, although *in vitro* evidence supports glutamate in this role. Because of this uncertainty, we performed a multitracer stable isotope study to determine whether proline, glutamate, or both are dietary precursors for arginine in enterally fed human neonates. Labeled arginine (M + 2), proline (M + 1), and glutamate (M + 3) were given enterally to 15 stable, growing preterm infants (GA at birth 30–35 wk) at 1–3 wk postnatal age. Enrichment in urine of the tracer amino acids and the M + 1 and M + 3 isotopomers of arginine were measured by LC-tandem mass spectrometry to determine the contribution of proline and glutamate to arginine synthesis. Plateau enrichments of arginine and proline tracers were measurable in urine. Urinary glutamate enrichment was not detected. Conversion of proline to arginine was detected. However, the M + 3 isotopomer of arginine, which would have been synthesized from glutamate, was not detected. We conclude that, in contrast to the current consensus in the literature based on *in vitro* studies, proline is the major contributor to arginine synthesis in human preterm infants. (*Pediatr Res* 69: 46–50, 2011)

In most mammals, including humans, arginine is considered a conditionally indispensable amino acid that may become an essential component of diet during times of maximal growth or critical illness, *e.g.* burn injury (1–3). An infant will receive ~45 mg/kg/d of arginine from human milk (4); however, assuming 2 g/kg/d net protein accretion, healthy neonates require at least 140 mg/kg/d for protein synthesis alone; therefore, a high rate of arginine synthesis is implied.

In infants requiring neonatal intensive care, it is clear that arginine is an essential dietary amino acid. This has been demonstrated by the observation that infants receiving total parenteral nutrition (TPN) deficient in arginine developed hyperammonemia that was responsive to arginine infusion (5,6). Infants in intensive care have hypoargininemia compared with healthy infants (7), and arginine deficiency has been investigated in the development of necrotizing enteroco-

litis (NEC) (8) and pulmonary hypertension (9). Furthermore, arginine supplementation was shown to reduce the incidence of all stages of NEC in moderately at risk infants (10) and a single bolus infusion of i.v. arginine improved oxygenation in infants with pulmonary hypertension (11). Therefore, because arginine is clearly important for metabolism in the neonate, it is critical to understand the metabolic pathways involved in its synthesis.

The process for arginine synthesis in the adult involves an intestinal-renal axis. Citrulline is synthesized in the enterocyte either from glutamate *via* P5C synthase or from proline *via* proline oxidase (12). Citrulline is then released to the portal venous system; it is not taken up by the liver but by the kidney (13) where it is converted to arginine *via* argininosuccinic acid using the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL; Fig. 1).

However, in neonatal mammals, the process of arginine synthesis takes place entirely within the gut; the enterocyte has low arginase in the neonatal period and the proximal tubule of the kidney, where citrulline is converted to arginine in the adult, has low ASS and ASL (7). When this mechanism of neonatal arginine synthesis converts to the intestinal-renal axis of adults or what mediates the change is not clear. It is also unclear whether proline or glutamate is the main precursor for arginine synthesis in the neonate because there are conflicts in the literature between the results reported using *in vivo* versus *in vitro* methods.

By using the neonatal pig model, our laboratory has shown that arginine is synthesized from enteral proline and is able to ameliorate an arginine deficient diet (14). Furthermore, we have shown that the ability of proline to act as a precursor for arginine is dependent on normal intestinal function (15). There was negligible synthesis of arginine from glutamate whether it was given enterally or parenterally (14,16). When fed an arginine-deficient diet, intestinal synthesis was increased but not at a rate sufficient to meet metabolic demands (16). Similarly, in piglets fed with a proline-deficient diet, arginine was converted to proline, but the arginine label was not detected in glutamate, although it was present in ornithine and citrulline (17). Conversely, *in vitro* work using piglet entero-

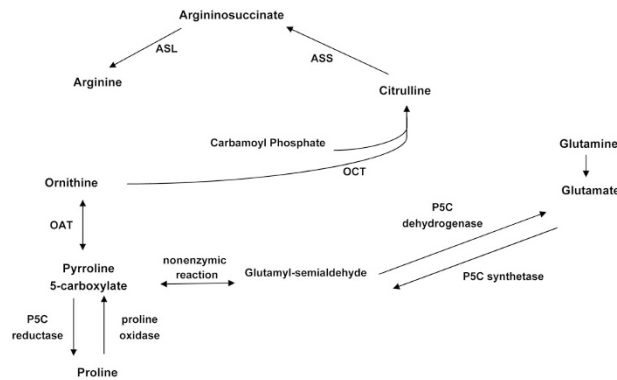
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**Abbreviations:** ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; MPE, mole percent excess



**Figure 1.** Pathway of arginine synthesis. ASL (EC–4.3.2.1), ASS (EC–6.3.4.5), OAT, ornithine aminotransferase (2.1.6.13), OCT, ornithine carbamoyl transferase (2.1.3.3), P5C reductase (1.5.1.2), proline oxidase (1.5.99.8), P5C dehydrogenase (1.5.1.12), and P5C synthetase (EC–not assigned).

**Table 1.** Patient details

	Mean	Range	SD
GA at birth (completed wks)	32.6	30–35	±1.5
Birth weight (kg)	1.75	1.0–2.16	±0.32
Age at study (d)	2.7	7–21	±1.8
Weight at study (kg)	1.85	1.48–2.25	±0.25
Weight change (kg)	0.1	–0.05–0.45	±0.18

n = 5.

cytes has shown that glutamate is a significant precursor of arginine (18). Recent studies in fasting adults also showed significant conversion of 2-<sup>15</sup>N glutamine to arginine and citrulline (19). However, the use of a <sup>15</sup>N-labeled tracer makes these results difficult to interpret because any of the nitrogen atoms in arginine may be derived ultimately from the amino nitrogen of glutamine or glutamate or both.

By using a multitracer stable isotope design, we sought to determine the ability of the human neonate to synthesize arginine and its immediate biochemical precursors, citrulline and ornithine, from the potential dietary precursors proline and glutamate.

## METHODS

**Subjects.** Fifteen low-birth weight infants were recruited from the nursery at St. Michael's Hospital, Toronto. Infants were eligible for the study if they were between 30 and 34 wk GA, <21 d old, and fully enterally fed with either formula or expressed breast milk. The infants were not receiving respiratory support or i.v. medications and had no congenital anomalies. The clinical characteristics of the infants are summarized in Table 1. At the time of the study, all infants were receiving 160 mL/kg/d of either expressed breast milk and fortifier or formula (Enfamil). Informed written consent was obtained from one or both parents. The study was approved by the Research Ethics Boards at The Hospital for Sick Children and St. Michael's Hospital, Toronto.

**Study design.** All infants received three labeled amino acids, which were given enterally either orally or *via* nasogastric (NG) tube. As all infants were fully fed and did not have venous access, it was not possible to use i.v. tracers. Infants were given a priming dose, and to simulate a constant infusion, this was followed immediately and subsequently by further equal boluses of labeled amino acids every 2 or 3 h depending on the feeding protocol (20). To ensure complete delivery of the labeled amino acids, they were given immediately before feeds through the NG tube or in a bottle nipple. As a surrogate for plasma, and to ensure the studies were as minimally invasive as possible, isotope enrichment was measured in urine, as previously validated (20,21). Enrichment reached plateau after 6 h as assessed by visual inspection of the

data and an absence of any slope. Urine was collected in bags at each diaper change prefeed and either two or three hourly depending on the feeding protocol. Urine was stored frozen at –20°C until analysis.

The doses of amino acid tracers were given to simulate a primed constant infusion as follows: [<sup>15</sup>N<sub>2</sub>-guanidino] arginine, 20 μmol/kg prime, followed by 40 μmol/kg/2 h bolus or 60 μmol/kg/3 h bolus; [<sup>15</sup>N] proline, 20 μmol/kg prime, followed by 40 μmol/kg/2 h bolus or 60 μmol/kg/3 h bolus; and [2,4,4-<sup>2</sup>H<sub>3</sub>] glutamate, 30 μmol/kg prime, followed by 60 μmol/kg/2 h bolus or 90 μmol/kg/3 h bolus. Each infant was studied once and the study lasted for 12 h.

**Isotopes.** Isotopes were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Three labeled amino acids were used: [<sup>15</sup>N<sub>2</sub>-guanidino] arginine (isotope enrichment 99.4%, D-isomer 0.27%), [<sup>15</sup>N] proline (98.7%, <0.5%), and [2,4,4-<sup>2</sup>H<sub>3</sub>] glutamate (97.1%, <0.5%).

Solutions of the tracer were prepared by the pharmacy, at The Hospital for Sick Children, in sterile water and stored at 4°C until use. The concentration of the solutions was arginine 30 mg/mL, proline 25 mg/mL, and glutamate 5 mg/mL. Solutions were confirmed to be sterile and pyrogen free before use.

**Analysis of enrichment and chiral separation.** Fifty microliters of urine were deproteinized by the addition of 500 μL of methanol followed by centrifugation at 13,000×g for 5 min. The supernatant was dried under N<sub>2</sub> and reconstituted in 0.1% formic acid. Isotope analysis was performed using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) operated in positive electrospray ionization mode. This was coupled to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). All aspects of the system operation were controlled using the Analyst NT version 1.4.1 software (Applied Biosystems/MDS Sciex). Maximum sensitivity for L and D arginine, citrulline, ornithine, proline, and glutamate was achieved by measuring product ions multiple reaction monitoring from the fragmentation of the protonated [M + H]<sup>+</sup> molecules of each amino acid. Only the enrichments of the L-isotopes of the amino acids were analyzed for this study. The [M + H]<sup>+</sup> precursor (parent) ion for each of the amino acids was m/z 175.1 for arginine, 116.1 for proline, 176.1 for citrulline, 133.1 for ornithine, and 148.1 for glutamate. The signal for the most abundant product (daughter) ions (m/z 70.1 for arginine, 70.1 for proline, 70.1 for citrulline, 70.1 for ornithine, and 84.1 for glutamate) were also optimized. Chiral separations were performed with a Chirobiotic T (teicoplanin) HPLC column, 25 cm × 4.6 mm, 5 μm (Astec, Whippany, NJ) using an isocratic gradient with 95:5 (10 mM NH<sub>4</sub>Ac, pH = 4.1):(2:1 MeOH:0.1% FA in ACN) buffer at 700 μL/min. The injection volume was 20 μL with an overall analysis time (injection to injection) of 35 min.

The labeled [<sup>15</sup>N<sub>2</sub>-guanidino] arginine has a parent ion with m/z 177.1 and a daughter ion with m/z 70.1. Labeled [<sup>15</sup>N] proline has a precursor ion with m/z of 117.1 and a product ion of 71.1. The respective ions for [2,4,4-<sup>2</sup>H<sub>3</sub>] glutamate are 151.1 and 87.1.

The appearance of the proline and glutamate tracers in arginine was determined by measuring the precursor or product m/z ion pair of 176/71 for proline and 178/73 for glutamate. Similarly, the conversion of the labeled tracers into citrulline was determined using the precursor or product m/z ion pair of 177.1/71 from proline and 179/73 from glutamate. The synthesis of ornithine from the tracers was assessed using the m/z ion pairs of 134/71 from proline and 136/73 from glutamate.

**Data analysis.** Isotope enrichment was expressed as mole percent excess (MPE) calculated as enrichment at plateau minus the background measurement at baseline.

As it was not possible by the given study design and ethical constraints to give i.v. tracers to measure whole body flux, the turnover of the enterally infused amino acids was calculated using the equation:

$$Q = i[(E_i/E_u) - 1],$$

where  $Q$  is the turnover of the amino acid through the free amino acid pool,  $i$  is the rate of infusion of the tracer,  $E_i$  is the enrichment of the tracer in the infusion, and  $E_u$  is the MPE of the tracer in urine at plateau.

The conversion rates of the infused isotopes to arginine was calculated by the formula

$$Q_{\text{precursor-product}} = E_{\text{product}}/E_{\text{precursor}} \times Q_{\text{arginine}}$$

The fractional conversion of the infused isotopes to ornithine and citrulline was calculated by the formula:

$$Q_{\text{precursor-product}} = E_{\text{product}}/E_{\text{precursor}}$$

**Statistical analysis.** Data are expressed as mean with SDs. Enrichment of amino acids above baseline was analyzed using Student's  $t$  test. Significance was assumed if  $p < 0.05$ . Statistical analysis was performed using SAS version 9.1 (SAS Institute, Cary, NC).

**Table 2.** Enrichment of labeled amino acids in plasma sampled in urine

	Mean MPE	SD	Enteral turnover ( $\mu\text{mol/kg/h}$ )	SD
( $^{15}\text{N}_2$ guanidino) Arginine	4.11	1.62	461	181
$1\text{-}^{15}\text{N}$ Arginine	0.7	0.9		
$2,4,4\text{-D}_3$ Arginine	ND	ND		
$1\text{-}^{15}\text{N}$ Citrulline	2.6	1.7		
$2,4,4\text{-D}_3$ Citrulline	ND	ND		
$1\text{-}^{15}\text{N}$ Ornithine	2.8	1.68		
$2,4,4\text{-D}_3$ Ornithine	ND	ND		
$1\text{-}^{15}\text{N}$ Proline	7.7	1.9	237	58
$2,4,4, \text{D}_3$ Glutamate	ND	ND	—	—

ND, not detected at MPE  $>0.1$  above baseline.

$\text{D}_3 = {}^2\text{H}_3$ .

## RESULTS

All infants completed the study, tolerated it well, and there were no adverse events. Enrichment was measured in urine of the isotopes for  $^{15}\text{N}_2$  arginine and  $^{15}\text{N}$  proline (Table 2). No enrichment was detected, in any infant, of the M + 3 [ $2,4,4, \text{D}_3$ ] glutamate; there was no rise in the occurrence of this isomer over background. The appearance of label from the proline tracer in ornithine, citrulline, and arginine was observed in all infants.

The mean enrichment for the arginine tracer was 4.11% ( $\pm 1.62\%$ ). This corresponds to an enteral turnover rate for arginine of 461  $\mu\text{mol/kg/h}$  ( $\pm 181 \mu\text{mol/kg/h}$ ). The enrichment for the proline tracer was 7.7% ( $\pm 1.9\%$ ) corresponding to a turnover rate of 237  $\mu\text{mol/kg/h}$  ( $\pm 58 \mu\text{mol/kg/h}$ ).

Table 2 shows the synthesis of arginine and its intermediaries, ornithine and citrulline, from the proline isotope. Synthesis of all three amino acids from proline was demonstrated with enrichment in ornithine of 2.8% (SD  $\pm 1.68\%$ ), citrulline of 2.6% (SD  $\pm 1.7\%$ ), and arginine of 0.7% (SD  $\pm 0.9\%$ ).

The fractional conversion of proline to ornithine was 0.36 and from proline to citrulline was 0.34. The rate of conversion of proline to arginine was 42  $\mu\text{mol/kg/h}$ . There was no M + 3, or M + 2, isomer of any of the three amino acids above baseline in any infant, indicating no evidence of synthesis of these amino acids from enteral glutamate.

## DISCUSSION

Given the low arginine content of human milk and implied high rate of arginine synthesis, the objective of this study was to document whether human infants are able to synthesize arginine and to clearly identify the dietary precursors. To our knowledge, this is the first multistable isotope study quantifying the metabolism of arginine in enterally fed human preterm infants. We have demonstrated that arginine and its intermediary metabolites, ornithine and citrulline, are synthesized from proline. Conversely, we have shown that there is no conversion *in vivo* of the carbon skeleton from dietary glutamate into arginine or its precursors as measured by this method. Clearly, in enterally fed human infants, proline is the major, if not the only, dietary precursor for arginine. This observation has significant implications when considering arginine requirements.

We know of no other potential dietary precursor for arginine; therefore, proline and arginine must be regarded as codependant, with arginine and proline requirements considered in reference to the sum of arginine and proline intake. Therefore, to the estimate of arginine requirements of 140 mg/kg/d (0.8 mmol), the proline requirement of 180 mg/kg/d (1.56 mmol) must be added (assuming a proline content of protein of 9% and 2 g/kg/d net protein synthesis), suggesting a total molar requirement for arginine plus proline of 2.4 mmol/kg/d. The sum of the molar content of arginine (0.26 mmol/g protein) and proline (0.74 mmol/g protein) in human milk is 1 mmol/g protein (22), which implies a protein intake of 2.4 g/kg/d to provide the estimated requirement of arginine and proline. Then, it follows that arginine or proline intake is likely inadequate during periods of the NICU stay as this level of protein intake for preterm infants cannot be met with human milk until full feeds are established and fully fortified. These observations suggest that the low arginine documented in preterm infants (7) relates to inadequate intake of proline and arginine. Whether this is clinically important with either short- or long-term effects is unknown.

The enteral turnover measurements of arginine and proline in our study are the first to be published for healthy growing preterm infants; therefore, it is necessary to discuss our results with reference to adult human studies and piglet studies as a model for the preterm infant. The arginine rate determined in our study (460  $\mu\text{mol/kg/h}$ ) is considerably higher than that calculated by Castillo *et al.* (9), who measured an arginine flux of 125  $\mu\text{mol/kg/h}$  by using an i.v. tracer in infants recovering from persistent pulmonary hypertension of the newborn. Given the substantial metabolism of arginine by the splanchnic organs, which is estimated to be as high as 50% in piglets (23) and between 31 and 38% in adult humans (24), the rate in our study was expected to be higher. However in the study by Castillo *et al.* (9), the infants were more mature, being born at term, were recovering from significant pulmonary hypertension, and were being fed smaller amounts of protein (1.2 g/kg/d) and energy (70 kcal/kg/d) when compared with this study in which infants were completely enterally fed receiving  $\sim 160 \text{ mL/kg/d}$  of milk.

Our estimate of turnover for enteral arginine of 460  $\mu\text{mol/kg/h}$  is higher than the value in adults, in whom the turnover rate of intragastric arginine was estimated to be  $\sim 120 \mu\text{mol/kg/h}$  (24). The higher rate in premature neonates compared with adults can be explained by the higher rate of endogenous protein turnover in newborns, which may be as much as 4–6 times greater than that of adults (25,26) and the increased requirement for arginine to meet the metabolic demands of growth.

The enteral proline turnover rate determined in our study is 237  $\mu\text{mol/kg/h}$  ( $\pm 58 \mu\text{mol/kg/h}$ ). There are no comparable data in either human infants or adults. The only available data in humans is i.v. tracer whole body flux rates from a series of studies by Jaksic *et al.* (27–29), who determined a flux of proline of 70–75 mol/kg/h in healthy adults. The ratio between enteral arginine turnover in premature infants (460  $\mu\text{mol/kg/h}$ ) and i.v. arginine flux in adults (120  $\mu\text{mol/kg/h}$ , 23) is 3.8:1. By comparison, the ratio of enteral proline

turnover in infants ( $237 \mu\text{mol/kg/h}$ ) to i.v. proline flux in adults ( $70 \text{ mol/kg/h}$ , 25–27) is 3.4:1. This similarity suggests that the pattern of metabolism of the two amino acids, *i.e.* rate of absorption and metabolism by splanchnic organs occurs in similar proportion. However, animal data would suggest that only a small proportion, at most 14%, of proline is extracted by the splanchnic organs (30) compared with a value of 50% for arginine. The similarity in this ratio for arginine and proline suggests that the splanchnic metabolism of proline in humans may be much higher than previously believed and deserves further investigation to elucidate the proline requirements for the splanchnic organs and whether it is required for protein synthesis alone, amino acid precursor, or other metabolic roles.

Of note, in our estimates of enteral turnover, for both arginine and proline, is the considerable variation among subjects, which is greater for arginine than proline reflecting the multiple metabolic roles for arginine, notably creatine and NO synthesis. The source of this variation is likely multifactorial. It is well recognized that protein turnover rates in preterm infants are much higher than adults and show considerable variation among individuals. For example, Denne *et al.* (25), in infants of similar weight to those in our study ( $1.55 \pm 0.34 \text{ kg}$ ), reported whole body protein synthesis rates of  $10.5 \text{ g/kg/d}$  but with a SD of  $3 \text{ g/kg/d}$ . Protein synthesis is the largest component of arginine flux, and therefore, this likely explains the variation observed in our study. In addition, and by necessity, it was not possible to standardize feeds in this study beyond feed volume, and therefore, variation in nutrient intake will have contributed to the overall variation. Infants in our unit are considered to be at full feeds at  $160 \text{ mL/kg/d}$ . The milk of choice is expressed breast milk to which fortifier is added when preterm infants reach full feeds. The amount of protein and arginine in breast milk also displays considerable variation. Montagne *et al.* (31) demonstrated that in the mature milk of mothers whose babies were born prematurely, the amount of protein varied by  $\sim 20\%$  with the variation being even greater when the infants were younger.

The arginine intake in infants who are fed breast milk has been estimated to be  $45 \text{ mg/kg/d}$  (4) notwithstanding the variation described above. This equates to an intake of  $\sim 10 \mu\text{mol/kg/h}$ . By comparison, our results indicate an arginine synthesis rate of  $44 \mu\text{mol/kg/h}$  from proline, indicating *de novo* arginine synthesis provides more arginine than is derived from breast milk and is equivalent to  $\sim 198 \text{ mg/kg/d}$ . This amount would account for the  $160 \text{ mg}$  of arginine per  $\text{kg/d}$  required to support protein synthesis and hence growth. This is also true in the piglet, in which arginine intake in sows' milk meets only  $\frac{1}{4}$  of the estimated metabolic requirement for arginine (32). There are no human data with which to compare the fractional synthesis rates of ornithine and citrulline; however, these are similar to the synthesis rates reported for the piglet (33).

We did not measure any glutamate tracer in the urine glutamate pool. Recognizing that this was a possibility because of high dilution from dietary and endogenous glutamate, we dosed more on a per kg body weight basis than the other tracers. Because glutamate has a relatively low solubility, we

estimated that this was the maximum dose of glutamate that could be given enterally to the infants without risk of inducing feed intolerance. Similarly, we chose the glutamate isotope as  $M + 3$ , which is present naturally at only low amounts and thus would allow us to detect lower levels of enrichment. It is possible that absorption of free intraluminal glutamate may have been incomplete; however, this is an unlikely explanation as enrichment of the arginine and proline tracers was significant, and in piglets, it has been demonstrated that almost all enteral glutamate is absorbed and subsequently metabolized by the small intestine (34). A further possible reason for the lack of enrichment of the glutamate pool is that of proton substitution. This happens when a labeled deuterium atom is replaced by an unlabeled proton. This is an unlikely explanation in this case because the isomer used was  $[2,4,4, D_3]$  glutamate, and these deuterium atoms are unlikely to be spontaneously replaced because of their location on the molecule. For example, studying deuterium exchange in arginine, Huang *et al.* (34a) demonstrated that this took place mostly at the carboxyl group and to a lesser extent on the amino N atom. By studying leucine metabolism using two tracers, one carbon the other deuterium labeled, Hoerr *et al.* (35) found no evidence of deuterium substitution. However, in case there was systematic proton substitution, we also looked for  $M + 2$  and  $M + 1$  isomers of glutamate but found no enrichment over baseline (data not shown). Despite the lack of evidence that deuterium substitution may be occurring, the use of a carbon-labeled tracer would circumvent the issue and also allow measurement of oxidation.

Notwithstanding these issues, we conclude that as there was no measurable enrichment of glutamate, it is clear that enterally supplied glutamate is entirely metabolized by the splanchnic organs on first pass. This is in keeping with the findings of others that there is a substantial metabolism of glutamate and glutamine by the gut (30,34,36).

Although there was no enrichment of the urine glutamate pool from the enteral glutamate tracer, if there had been significant synthesis of arginine and intermediaries from glutamate in the splanchnic tissues, we would have detected  $M + 3$  isomers of these amino acids. However, no  $M + 3$  isomers of arginine, ornithine, or citrulline were detected above baseline in any infant. Moreover, to ensure no systematic deuterium substitution,  $M + 2$  isomers of arginine, citrulline, and ornithine were looked for but not found (data not shown). Therefore, although it is clear that P5C synthase is present in piglet enterocytes (18) and that arginine may be synthesized from glutamine in isolated enterocytes (18), there is negligible, or undetectably low, synthesis of arginine from dietary glutamate in healthy growing preterm infants *in vivo*, despite the significant metabolism of glutamate by the splanchnic organs. We hypothesize that in the neonatal enterocyte *in vivo* that the carbon chain of glutamate is used primarily as an energy source and is rapidly oxidized (36) with the nitrogen atom either excreted or used through transamination.

The main finding in our study is that the dietary precursor for arginine is proline, with no synthesis from glutamate. This is consistent with our work using the neonatal pig in which we have demonstrated, using a number of dietary approaches

(14,15,37,38), that arginine can only be synthesized from proline through citrulline and that there is no *in vivo* synthesis from enteral glutamate.

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