

Dietary Arginine Requirements for Growth Are Dependent on the Rate of Citrulline Production in Mice^{1–3}

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Abstract

Background: In many species, including humans, arginine is considered a semiessential amino acid because under certain conditions endogenous synthesis cannot meet its demand. The requirements of arginine for growth in mice are ill defined and seem to vary depending on the genetic background of the mice.

Objective: The objective of this study was to determine the metabolic and molecular basis for the requirement of arginine in 2 mouse strains.

Methods: Institute of Cancer Research (ICR) and C57BL/6 (BL6) male mice were fed arginine-free or arginine-sufficient diets (Expt. 1) or 1 of 7 diets with increasing arginine concentration (from 0- to 8-g/kg diet, Expt. 2) between day 24 and 42 of life to determine the arginine requirements for growth. Citrulline production and "de novo" arginine synthesis were measured with use of stable isotopes, and arginine requirements were determined by breakpoint analysis and enzyme expression by reverse transcriptase-polymerase chain reaction.

Results: In Expt. 1, ICR mice grew at the same rate regardless of the arginine concentration of the diet (mean \pm SE: 0.66 \pm 0.04 g/d, $P = 0.80$), but BL6 mice had a reduced growth rate when fed the arginine-free diet (0.25 \pm 0.02 g/d, $P < 0.001$) compared to the 8-g arginine/kg diet (0.46 \pm 0.03 g/d). ICR mice showed at least a 2-fold greater expression ($P < 0.001$) of ornithine transcarbamylase (*OTC*) than BL6 mice, which translated into a greater rate of citrulline (25%) and arginine synthesis (49%, $P < 0.002$). In Expt. 2, breakpoint analysis showed that the requirement for growth of BL6 mice was met with 2.32 \pm 0.39 g arginine/kg diet; for ICR mice, however, no breakpoint was found.

Conclusion: Our data indicate that a reduced expression of *OTC* in BL6 mice translates into a reduced production of citrulline and arginine compared with ICR mice, which results in a dietary arginine requirement for growth in BL6 mice, but not in ICR mice. *J Nutr* 2015;145:1227–31.

Keywords: amino acid, arginine, citrulline, growth, requirements

Introduction

Arginine is considered a semiessential amino acid because, during certain physiologic and pathophysiologic conditions, endogenous synthesis cannot meet the demand for this amino acid. The endogenous synthesis of arginine is a multiorgan process that involves the synthesis of citrulline by the gut and further conversion into arginine by the kidney (1), where argininosuccinate synthase and lyase (enzymes that catalyze

the conversion of citrulline into arginine) are highly expressed. In addition, these 2 enzymes are expressed in multiple tissues, and it is likely that a fraction of the citrulline produced is used directly by the tissues to meet, at least partially, their arginine needs (2, 3).

The synthesis of arginine shows marked species differences [for a review see Ball et al. (4)]. Arginine is an essential amino acid in birds because of the lack of carbamoyl phosphate synthase I (CPS I)⁶ (5), the enzyme that catalyzes the synthesis of carbamoyl phosphate, which becomes the ureido group of citrulline. In felines, it seems that the inability to synthesize citrulline is due to reduced ornithine aminotransferase activity, and thus, to the low availability of ornithine, the immediate

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³ Supplemental Figures 1 and 2 and Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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⁶ Abbreviations used: BL6, C57BL/6; CPS I, carbamoyl phosphate synthase I; ICR, Institute of Cancer Research; OTC, ornithine transcarbamylase.

TABLE 1 Fluxes and amino acid concentrations of BL6 and ICR mice fed arginine-sufficient (8-g arginine/kg diet) and arginine-free diets¹

	BL6		ICR		Gen	TRT	Gen × TRT
	Arg−	Arg+	Arg−	Arg+			
Fluxes, $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$							
RaPhe ²	330 ± 10	336 ± 16	326 ± 14	320 ± 17	0.52	0.99	0.71
RaTyr ²	298 ± 10	294 ± 14	276 ± 10	303 ± 12	0.62	0.43	0.29
RcPhe → Tyr ²	58 ± 5	55 ± 5	47 ± 3	68 ± 11	0.86	0.17	0.09
Plasma concentrations, $\mu\text{mol/L}$							
Citrulline	124 ± 5	126 ± 11	170 ± 5	146 ± 7*	0.001	0.23	0.16
Arginine	124 ± 8	165 ± 18	150 ± 16	147 ± 10	0.83	0.29	0.23
Ornithine	103 ± 11	109 ± 12	162 ± 4	131 ± 5*	0.002	0.31	0.14

¹ Values are means ± SEMs, $n = 10$. *Different from corresponding Arg−, $P < 0.05$. BL6, C57BL/6; Gen, genotype; ICR, Institute of Cancer Research; TRT, treatment.

² RaPhe, RaTyr, and RcPhe → Tyr: rate of appearance of phenylalanine and tyrosine, and rate of conversion of phenylalanine into tyrosine.

citrulline precursor (6). In other species, such as the rat and the pig, citrulline production is limited and endogenous arginine synthesis is not sufficient to allow for maximal growth (7, 8). In fact, the rate-limiting step in the endogenous synthesis of arginine is citrulline availability, and for this reason citrulline supplementation has been shown to successfully address arginine deficiency in multiple species [birds (9, 10), cats (11), pigs (12), rats (13)].

In mice, early observations indicated that this species was able to synthesize arginine (14), but it was not clear if this endogenous source of arginine was sufficient to meet the demand for this amino acid. Current dietary arginine recommendations [3-g/kg diet (15)] are based on the work of John and Bell (16, 17). However, these researchers could not establish arginine requirements because growth was not affected, even at the lowest arginine concentrations tested [1 g/kg (17) and 3 g/kg (16)]. In their work, John and Bell used Swiss × Carworth Farms No. 1 crossbred mice (both outbred lines) that have a high postweaning growth rate (~0.8 g/d). Our work with Institute of Cancer Research (ICR) mice, an outbred line also derived from Swiss mice, agrees with these previous observations and indicates that these mice may not have an arginine requirement. This contrasts with our observations in C57BL/6/J mice, a widely used inbred strain and the most common genetic background for genetically modified mice, in which growth was compromised when arginine-free diets were fed.

The objective of the present work was to elucidate the basis of arginine requirements in 2 mouse lines and to determine their arginine requirements for growth after weaning.

Methods

Mice and housing. Twenty-one-day-old male C57BL/6 (BL6) J0LaHsd mice and ICR [Hsd:ICR (CD-1)] mice were purchased from Harlan Laboratories. Upon arrival (day 21) mice were weighed, caged in pairs, and fed an irradiated 18% crude protein feed (Rodent Diet 2920×; Harlan Teklad). Dietary proximate analysis was as follows: protein (185 g/kg), arginine (8 g/kg), gross energy (14.1 MJ/kg), fat (60 g/kg), fiber (28 g/kg), and ash (46 g/kg). This pelleted diet was ground using a blender (Waring), and mice consumed the diet ad libitum using jar feeders for 3 d. After this acclimatization period (day 24), mice were fed the different diets described below until week 6 of life (day 42). Mice were weighed twice weekly between 1000 and 1200. Mice were under a 12-h light cycle (0600 to 1800) in a temperature-controlled ($22 \pm 2^\circ\text{C}$) and humidity-controlled ($55 \pm 5\%$) environment. Autoclaved reverse osmosis water was available at all times and the bedding used was corn cobs. All animal procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Expt. 1. BL6 and ICR mice ($n = 10$) were fed an arginine-sufficient (8-g/kg diet) or arginine-free purified diet based on the Rodent Diet 2920× (Table 1) from day 24 to day 42. At the end of feeding trial, after 4 h of feed deprivation, mice were infused with stable isotopes to determine the rate of appearance of arginine, citrulline, and the contribution of citrulline to plasma arginine ("de novo" arginine synthesis). Phenylalanine and tyrosine tracers were also used to determine whole-body protein turnover. Methods and calculations have been described in detail elsewhere (18, 19). The plasma concentrations of arginine and citrulline were determined by isotopic dilution with use of an LC-MS/MS (TSQ Vantage; Thermo Scientific).

Expt. 2. To determine the arginine requirements of BL6 and ICR mice for growth ($n = 10$) between day 24 and day 42, diets with different arginine concentrations were fed. By mixing the arginine-free diet and arginine-sufficient diet (Supplemental Table 1), diets containing 0-, 0.6-, 1-, 1.7-, 3-, 5-, and 8-g arginine/kg diet were obtained.

Expt. 3. To determine the expression of CPS I and ornithine transcarbamylase (OTC), enzymes that catalyze the synthesis of citrulline in the small intestine, day 24, day 32, and day 42 mice ($n = 5$) were killed. Mice were fed Rodent Diet 2920×. Gut tissue was snap frozen immediately. Tissue was homogenized in guanidinium thiocyanate-phenol-chloroform extraction (TRIzol; Life Technologies), and RNA was isolated with use of Qiagen RNeasy Mini elute columns (Qiagen). After cDNA synthesis, PCR amplification and gene differentiation expression (primers are shown in Supplemental Table 2) were performed with SYBR Green1 on a multicolor real-time PCR detection system (CFX96; BioRad Laboratories).

Data analysis. Data were analyzed statistically with use of the proc mixed procedure of SAS (v. 9.2; SAS Institute) with arginine concentration, genetic background, and their interaction as the fixed effect of the model (20). mRNA expression data were log transformed before

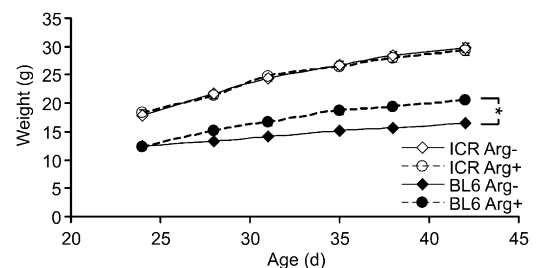


FIGURE 1 Growth curves of BL6 and ICR mice fed arginine-free or arginine-sufficient diets (8-g/kg diet). Symbols are means ± SEs, $n = 10$. *Difference in growth rate within genotype, $P < 0.05$. BL6, C57BL/6; ICR, Institute of Cancer Research.

analysis; genotype and age were the fixed effects of the model. Preplanned orthogonal contrasts were used to determine the effect of dietary arginine within genotype. Arginine requirements were determined by broken line regression with use of the proc NLIN statement of SAS (21). Values presented in the text are means \pm SEs and were tested for significance at the 5% level.

Results

ICR mice were heavier than BL6 mice (12.1 ± 0.20 g vs. 8.7 ± 0.11 g, $P < 0.001$) at weaning (day 21) and displayed a greater growth rate between day 24 and day 42 when fed the arginine-sufficient diet (0.66 ± 0.04 g/d and 0.46 ± 0.03 g/d, $P < 0.001$). Although ICR mice grew at the same rate regardless of the arginine concentration of the diet ($P = 0.80$), BL6 mice showed a reduction in growth rate when fed the diet devoid of arginine (0.46 ± 0.03 g/d vs. 0.25 ± 0.02 g/d, $P < 0.001$; **Figure 1**).

ICR mice had a faster rate of citrulline production ($P < 0.002$; **Figure 2A**) than BL6 mice, which translated into a greater “de novo” arginine synthesis ($P < 0.001$; **Figure 2B**). This increase, however, did not result into a higher flux of arginine ($370 \pm 18 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, $P > 0.40$). Likewise, no differences in the appearance rate of phenylalanine and tyrosine or in the rate of conversion of phenylalanine into tyrosine were observed for genotype ($P > 0.17$) or arginine concentration of the diet ($P > 0.52$; **Table 1**). ICR mice had higher plasma concentrations of ornithine and citrulline than their BL6 counterparts ($P \leq 0.002$; **Table 1**), and plasma arginine concentration was increased ($P < 0.05$) in BL6 mice consuming the arginine-sufficient diet (**Table 1**).

Arginine concentration of the diet had no effect ($P = 0.53$) on the growth curve of ICR mice (**Supplemental Figure 1**), but low

concentrations of arginine reduced growth in BL6 mice ($P < 0.008$; **Supplemental Figure 2**). For this reason no breakpoint for weight gain was found for ICR mice, which indicates that this strain does not require dietary arginine for growth (**Figure 3A**). BL6 mice, however, showed a clear breakpoint and thus a dietary requirement (2.32 ± 0.39 g arginine/kg) to maximize weight gain (**Figure 3A**). Thus, the dietary arginine needed to meet the requirements of 95% of the population was calculated as 3.1-g arginine/kg.

Feed intake for the 18-d period studied was greater for ICR mice than for the BL6 mice ($P < 0.001$; **Figure 3B**). Although, we were not able to detect differences in feed intake for the mice fed the different diets, a trend ($P = 0.06$) for a reduced intake was observed for the BL6 mice consuming diets with <1 g arginine/kg (**Figure 3B**).

The expression of *CPS I* in the small intestine was not different ($P = 0.88$) between BL6 and ICR mice; however, there was an age effect ($P < 0.006$) with a reduction by 42 d in the ICR mice (**Figure 4A**). A greater expression of *OTC* in ICR mice ($P < 0.001$) was observed at all ages; the 2-fold increased expression of *OTC* measured in ICR mice on 24 and 32 d increased ($P < 0.001$) to a 7-fold difference by 42 d (**Figure 4B**).

Discussion

Arginine is an amino acid used not only for protein synthesis, but also for the synthesis of creatine, polyamines, and nitric oxide and thus has a central role in energy metabolism, cell proliferation, and the regulation of blood pressure and the immune response. We chose to determine arginine requirements for growth because of the high demand of this amino acid for tissue deposition that takes place after weaning. Additionally, the difficulties and variability involved in determining the response of other physiologic and pathophysiologic endpoints to relatively small changes in dietary arginine make the task of estimating requirements for these additional endpoints daunting. Therefore, the implicit assumption in the determination of arginine requirements is that, under normal conditions, once the requirements for growth

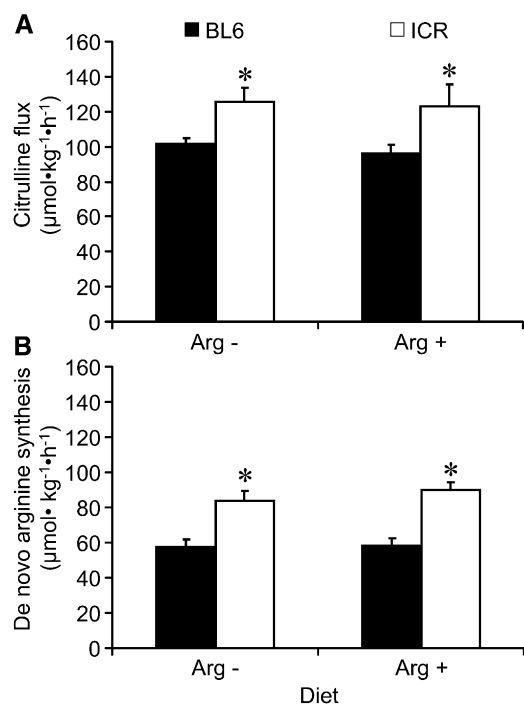


FIGURE 2 Citrulline flux (A) and de novo arginine synthesis (B) of BL6 and ICR mice fed arginine-free or arginine-sufficient diets (8-g/kg diet). BL6 mice had a reduced citrulline flux and de novo arginine synthesis than ICR mice ($P < 0.002$). Diet had no effect on these variables. Bars are means \pm SEs, $n = 10$. *Different from BL6 mice fed the same diet, $P < 0.01$. BL6, C57BL/6; ICR, Institute of Cancer Research.

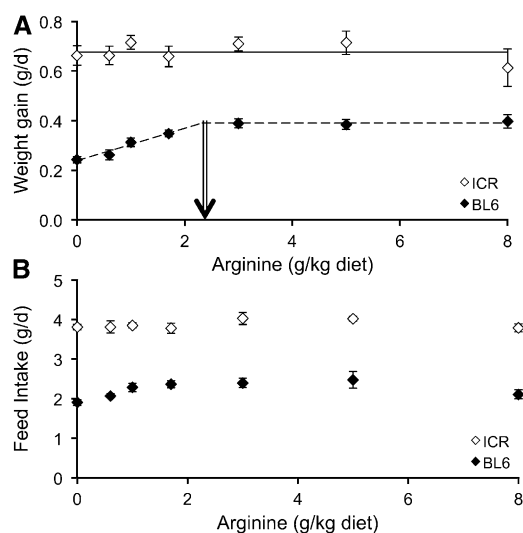


FIGURE 3 Daily weight gain (A) and feed intake (B) of BL6 and ICR mice fed diets containing different amounts of arginine. No breakpoint was identified for ICR mice. The arrow denotes the breakpoint for the BL6 mice (2.32 ± 0.39 g arginine/kg). Symbols are means \pm SEs, $n = 10$. BL6, C57BL/6; ICR, Institute of Cancer Research.

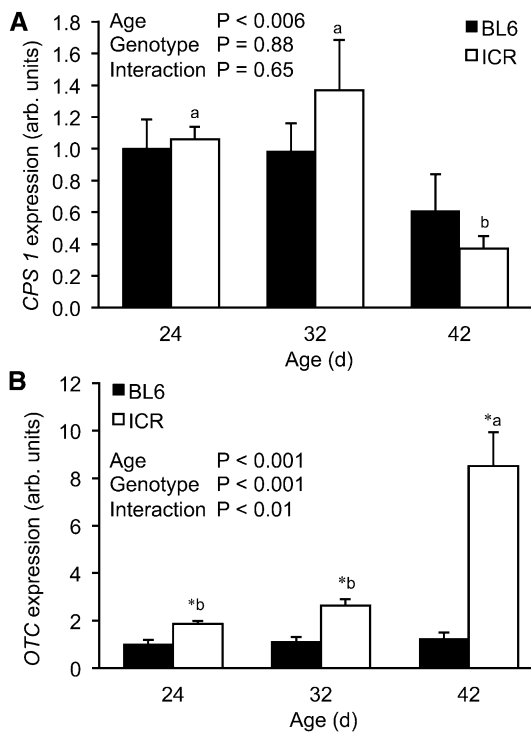


FIGURE 4 *CPS 1* (A) and *OTC* (B) mRNA expression in the small intestine of BL6 and ICR mice fed regular chow at different ages. *Different from BL6 mice at that age, $P < 0.05$. Within a genotype, labeled means without a common letter differ, $P < 0.05$. arb., arbitrary; BL6, C57BL/6; *CPS 1*, carbamoyl phosphate synthase I; ICR, Institute of Cancer Research; *OTC*, ornithine transcarbamylase.

are met, the requirements for the multiple functional roles of this amino acid are also met.

Previous attempts to determine requirements in mice indicated that this species had no arginine requirements for growth or that they were below the lowest arginine concentration fed (16, 17). Milner et al. (22), however, showed that mice fed an arginine-free diet resulted in feed intake depression and a reduction in body weight gain. These observations can be reinterpreted in light of the results we obtained. Although John and Bell (16, 17) used mice resulting from a cross between 2 outbred Swiss-derived mouse lines, Milner et al. (22) used mice derived from a cross between 2 inbred lines (BDF mice, C57BL/6 \times DBA/2). In our studies, we used outbred ICR mice (a line derived from Swiss strains) and inbred C57BL/6 mice. Paradoxically, the Swiss-derived lines with a higher growth potential (~ 0.8 g/d) and presumably a higher need for arginine did not show growth depression when fed arginine-free diets, whereas BL6 mice or their crosses, with a more modest growth rate (~ 0.4 – 0.5 g/d), suffered an $\sim 50\%$ reduction in their growth rate. The difference in the production of citrulline by the 2 genotypes may explain these findings because it can account for enough arginine to support ~ 0.20 g weight gain/d (see Appendix A). We previously reported similar genetic background differences in the production of citrulline between ICR and mixed-background mice (B6EiC3sn, the product of a C57BL/6J subline, and C3H/HeSnJX) (23). A similar difference in plasma citrulline and citrulline production between C57BL/6J and FVB mice (an inbred Swiss-derived strain) has been reported by others (24).

Not surprisingly, we did not find a growth rate breakpoint in ICR mice because these mice performed similarly at all the arginine concentrations fed. BL6 mice, however, showed a breakpoint at 2.3-g arginine/kg diet, which indicates the arginine

requirement for this strain. Thus, our present results confirm published observations that suggested that C57BL/6 mice and their crosses, but not mice from Swiss lineage, require arginine for growth. It seems that in the process of inbreeding to establish the C57BL/6 strain a reduced capacity for citrulline production was established. This is supported by the *OTC* expression data that indicates that this key enzyme for the synthesis of citrulline has a lower expression in BL6 mice than in ICR mice.

In conclusion, the variability in the expression of *OTC* resulted in a reduction in the production of citrulline and endogenous synthesis of arginine, which translated in the need for dietary arginine to meet the requirements for growth. Variability in the processes that result in the disposal of arginine (e.g., arginase) can further modify the requirement of a particular strain for arginine. Thus, these observations within a particular species described in this article recapitulate to a certain extent what has been known regarding the dietary need for arginine of different species (4). The first implication of our study is for those species in which arginine is required for maximal growth (e.g., neonatal pig). In these species, it is possible that enough genetic variability for citrulline production may be present and selecting for this trait may lead to an increase in endogenous arginine synthesis and a reduced need for dietary arginine. The second implication is that arginine is an essential amino acid for growth at least in some strains of mice. Thus, the requirements determined in a strain may not be valid for mice with a different genetic background. Current recommendations [3-g arginine/kg diet (15)] are able to meet the requirements for growth of C57BL/6 mice. Commonly fed diets, however, contain at least 8 g arginine/kg and are likely to meet the requirements under most, if not all, physiologic conditions.

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JCM designed and conducted the research, analyzed the data, wrote the paper, and has primary responsibility for final content; UA analyzed samples and reviewed the article; and ICD conducted the research and prepared samples for analysis. All authors read and approved the final manuscript.

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Appendix A

Here, we attempt to quantify the contribution of endogenous and dietary arginine to protein deposition and growth of BL6 mice.

Contribution of endogenous arginine. The measured production of citrulline in ICR and BL6 mice was 124 and 98 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, respectively. If BL6 mice were able to

sustain the same level of citrulline production as their ICR counterparts, it would amount to an extra 624- μmol citrulline $\cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (26 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \times 24 \text{ h/d}$). Because the only known fate for citrulline is its conversion into arginine, we expect this difference to correspond to an endogenous production of 109-mg arginine $\cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (624 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \times 174 \text{ mg/mmol}$). Therefore, for a 17-g mouse, this represents ~ 1.9 -mg arginine/d.

The arginine concentration of protein is 4.4-g arginine/100-g protein (1), and thus, 1.9-mg arginine/d if used for protein synthesis represents 42-mg protein/d. Finally, protein is $\sim 19\%$ of weight gain (2), and thus, it translates into 0.22-g weight gain/d.

Contribution of dietary arginine. The calculated arginine requirement (2.3-g arginine/kg diet) at the observed feed intake (2.2 g/d) provides 5.1-mg arginine/d. Arginine undergoes a high first pass extraction [75% (3)], which reduces the amount of arginine available to the peripheral tissues (1.3-mg arginine/d). Performing similar calculations as above, this represent a 0.15-g weight gain/d.

Although the implicit assumption of 100% efficiency in the use of arginine for protein synthesis is unlikely, a very high efficiency in the present scenarios (in which arginine is limiting) is reasonable. The first pass extraction rate used for these calculations was determined in mice fed an arginine-sufficient diet, and it is likely that in conditions of arginine deficiency the actual value is lower. This also assumes that first pass extraction implies a loss of arginine and ignores the use of arginine for protein synthesis (and other functions). Thus, the 0.15-g weight gain/d calculated is likely to be an underestimation. However, both calculations (contribution of endogenous and dietary arginine) give similar values that are remarkably close to the reduction in weight gain observed in the mice fed the arginine-free diet (0.21 g/d).

References (for Appendix A)

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