Amino Acid Supplementation Does Not Alter Whole-Body Phenylalanine Kinetics in Arabian Geldings

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Abstract

Stable isotope infusion methods have not been extensively used in horses to study protein metabolism. The objectives were to develop infusion and sampling methodologies for [1-13C] phenylalanine and apply these methods to determine whether the addition of supplemental amino acids to a control diet affected whole-body phenylalanine kinetics in mature horses. Arabian geldings were studied using a 6-h primed (9 μmol/kg), constant (6 μmol·kg⁻¹·h⁻¹) i.v. infusion of L-[1-13C] phenylalanine, with blood and breath sampled every 30 min, to measure whole-body phenylalanine kinetics in response to receiving the control diet (n = 12) or the control diet supplemented with equimolar amounts of glutamate (+Glu; 55 mg·kg⁻¹·d⁻¹; n = 5), leucine (+Leu; 49 mg·kg⁻¹·d⁻¹; n = 5), lysine (+Lys; 55 mg·kg⁻¹·d⁻¹; n = 5), or phenylalanine (+Phe; 62 mg·kg⁻¹·d⁻¹; n = 6). The plasma concentrations of the supplemented amino acid in horses receiving the +Leu, +Lys, and +Phe diets were 58, 53, and 36% greater, respectively, than for the control treatment (P < 0.05). Isotopic plateau was attained in blood [1-13C] phenylalanine and breath 13CO₂ enrichments by 60 and 270 min, respectively. Phenylalanine flux (+20%) and oxidation (+110%) were greater in horses receiving the +Leu, +Lys, and +Phe diets than in those fed the control diet. There was no effect of treatment diet on nonoxidative phenylalanine disposal or phenylalanine release from protein breakdown. The developed methods are a valuable way to study protein metabolism and assess dietary amino acid adequacy in horses and will provide a useful tool for studying amino acid requirements in the future. J. Nutr. 142:461–469, 2012.

Introduction

Compared to other domestic animals, little is known about the protein and amino acid requirements of horses of any age. The most recent edition of the Nutrient Requirements of Horses (1) provides requirements for only total crude protein and lysine; the latter was extrapolated to be 4.3% of the crude protein requirement. One approach to ensuring that adequate, but not excessive, amounts of the indispensable amino acids are met is to provide relatively high amounts of dietary crude protein; however, this is expensive and there are environmental consequences associated with the excretion of excess nitrogen (2). To optimize equine diets to ensure that adequate, but not excessive, amounts of the indispensable amino acids and protein are fed, a better understanding of whole-body protein metabolism and the requirements for those indispensable amino acids most likely to be limiting in equine diets is needed.

Recently, the WHO identified the IAAO9 method as an appropriate method for determining protein and amino acid requirements in humans (3). This method is based on the principle that indispensable amino acids are partitioned between protein synthesis and oxidation; if an amino acid cannot be used for protein synthesis because another amino acid is limiting to protein synthesis, then it is oxidized to CO₂ (4). The indicator amino acid, typically phenylalanine, is an indispensable amino acid other than the one that is limiting to protein synthesis and its oxidation to CO₂ is measured (4). Differences in phenylalanine oxidation can be measured through the primed, constant infusion of [1-13C] phenylalanine, with the measurement of the appearance of 13C in the exhaled CO₂ as protein synthesis.

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3 Supplemental information about the method development, Supplemental Tables 1 and 2, and Supplemental Figure 1 are available from the “Online Supporting Material” link in the online posting of the article from the same link in the online table of contents at jn.nutrition.org.
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7 Abbreviations used: DE, digestible energy; +Glu, glutamate-supplemented diet; IAAO, indicator amino acid oxidation; +Leu, leucine-supplemented diet; +Lys, lysine-supplemented diet; +Phe, phenylalanine-supplemented diet.
increases, the amount of $^{13}$C in the exhaled breath decreases until the point where the limiting amino acid is provided at its required level and is no longer limiting to protein synthesis (4). The appearance of [1-$^{13}$C]phenylalanine in the blood and tissues, including muscle, can be measured and used to calculate whole-body and tissue rates of protein synthesis, respectively (5,6). The IAAO method has also been used to identify limiting amino acids in the diet of parenterally fed piglets (7). These isotopic methods have the potential to greatly increase our understanding of protein and amino acid requirements and metabolism of horses of a variety of ages. However, these specific stable isotope techniques have not been previously applied in horses and some initial development of the procedures is necessary.

There are numerous commercial amino acid supplements for horses; however, it is unknown whether the addition of amino acids to a diet that already meets the NRC crude protein requirement (1) will result in any changes in whole-body protein metabolism. Lysine is the first limiting amino acid in the diets of horses; however, it is unknown whether the addition of amino acids to a diet that already meets the NRC crude protein requirement (1) will result in any changes in whole-body protein metabolism. Lysine is the first limiting amino acid in the diets of horses of a variety of ages. However, these specific stable isotope techniques have not been previously applied in horses and some initial development of the procedures is necessary.

The first objective of this study was to optimize the constant infusion procedures for isotopically labeled phenylalanine. It was hypothesized that determining the optimal prime:constant ratio for phenylalanine would result in the rapid attainment of isotopic plateau in blood and breath samples. The second objective was to use these isotopic procedures to determine whether whole-body phenylalanine kinetics were affected by the addition of equimolar amounts of lysine, leucine, phenylalanine, or glutamate to a control diet. If any of these amino acids were limiting to protein synthesis, we hypothesized that phenylalanine utilization for protein synthesis would be improved through the addition of the amino acid to the control diet.

Materials and Methods

The Virginia Tech Institutional Animal Care and Use Committee approved all procedures used in this study (08-080-APSC). All horses were obtained from the Middleburg Agricultural Research and Extension Center herd, were clinically healthy, and were on regular farrier, anthelmintic treatment, and vaccination (Eastern and Western encephalomyelitis, tetanus, rhinopneumonitis, influenza, West Nile virus, and rabies) schedules.

Animals and housing

Twelve adult (9–22 y) Arabian geldings of moderate to fleshy body condition (body weight $= 473 \pm 49$ kg, body condition score $= 6.7 \pm 0.9$) were used in this study. To control feed intake and ensure that horses were consuming only the study diets, horses were group-housed in a drylot paddock. Horses were bred into individual 3.7-×3.7-m stalls bedded with pine shavings for meal feeding, blood sampling, and isotope infusion procedures.

Feeding procedures

All horses were individually fed in stalls at 0700, 1400, and 1900 h daily, with the exception of isotope infusion days, when the feeding schedule was modified as described below. Horses received 12.5 g·kg$^{-1}$·d$^{-1}$ of a complete pelleted diet (Senior Pelleted Feed, Buckeye Nutrition) (Supplemental Tables 1 and 2) formulated to meet or exceed all nutrient requirements for mature, idle horses (1). To ensure that adequate DE was provided without providing excessive crude protein, the pelleted feed was top-dressed with 0.27 mL·kg$^{-1}$·d$^{-1}$ of corn oil. The combination of the complete pelleted diet with the oil was the control diet. The control diet was the base for all of the amino acid-supplemented diets, with the individual amino acids added to the pellets and oil immediately before feeding. Horses received one-third of their daily feed, corn oil, and supplemental amino acid allocation at each meal and had continuous access to water and a salt block. On d 8 and 9 of each study period, the 0700 h meal was divided into 16 equal portions and 1 portion of the meal was fed every 30 min beginning 90 min prior to the start of isotope infusion procedures and continuing throughout the infusions. Feeding small, frequent meals was done to ensure that horses were at a steady metabolic state during isotope infusions, so that steady-state isotope kinetics could be used (18).

Study design and procedures

Each horse was studied during three 9-d study treatment periods. In period 1, all horses received the control diet (control), and in each of periods 2 and 3, horses were randomly allocated to receive one of four amino acid-supplemented diets (n = 6/diet). Horses were allocated to the different amino acid-supplemented diets such that no horse received the same amino acid-supplemented diet twice and there was an equal number of horses receiving each supplemented diet in each period (n = 3/treatment each period). The supplemented amino acids were lysine (+Lys), leucine (+Leu), phenylalanine (+Phe), and glutamate (+Glu) (Sigma Aldrich). The +Lys treatment provided 55 mg·kg$^{-1}$·d$^{-1}$lysine, which is equivalent to the daily lysine requirement for a mature, idle horse (1). The amount of leucine (49 mg·kg$^{-1}$·d$^{-1}$), phenylalanine (62 mg·kg$^{-1}$·d$^{-1}$), and glutamate (55 mg·kg$^{-1}$·d$^{-1}$) supplemented to the respective daily diets was isomolar to the lysine supplementation. The +Glu treatment was included in this study to determine whether the control diet was limiting in amino nitrogen rather than a specific amino acid. Additionally, because lysine contains two nitrogen atoms, an additional 55 mg·kg$^{-1}$·d$^{-1}$ of glutamate was added to the +Leu, +Phe, and +Glu diets to ensure that all amino acid-supplemented diets were isonitrogenous.

Each 9-d period followed the same study timeline. On the afternoon of d 0, horses were weighed on a calibrated electronic scale (GSE Scale Systems) and allocated to one of the study diets. Horses were adapted to these diets for 7 d. In humans (19) and pigs (20), <2 d of adaptation is required prior to using the IAAO methodology to study whole-body amino acid requirements and metabolism; however, because similar studies have not confirmed this in horses, an adaptation period of 7 d was used. On the morning of d 7, horses were weighed and catheters (14 gauge × 14.0 cm, Abboctah; Abbott Laboratories) were inserted using an aseptic technique into each jugular vein, one for isotope infusion and a second for blood sampling. To determine the effect of treatment on plasma amino acids, blood samples (~10 mL) were collected before (t = 0) and 120 min after the 1400 h meal on d 7. Previous research in horses has shown that plasma amino acid concentrations reach peak concentrations between 1.5 and 3 h following a meal (21).

Each horse received a 6-h primed (9 μmol/kg), constant (6 μmol·kg$^{-1}$·h$^{-1}$) infusion of 1-[1-$^{13}$C]phenylalanine (m+1; Cambridge Isotopes) on the morning of d 8 to measure whole-body phenylalanine kinetics (6). The prime:constant ratio of 1.5 was based on results from a preliminary study (see Supplemental Material for a complete explanation and summary of results). The stable [1-$^{13}$C]phenylalanine solution was made by dissolving the isotope in 0.9% sterile saline and then filtering the solution through a 0.22-μm sterile filter (Millipore) into a sterile ethylene vinyl acetate bag (Baxter Healthcare). Isotope was delivered using a pressure-sensitive, cordless, i.v. infusion pump (VetIV, Heska), which was attached to each horse using a surcingle. Blood (10 mL) and breath (~1 L) samples were taken at ~30 (baseline), 0 (baseline), 30, 60, 90, 120, 150, 180, 210, 240, 300, 330, and 360 min after the start of the constant infusion. Breath samples were collected using an Equine Aeromask (Trudell Medical International) that was...
modified by reversing one of the 1-way valves to allow air to be trapped in gas-impermeable bags (Wagner Analysen Technik Vertriebs). When collecting breath samples, horses wore the breath collection masks for a minimum of 1 min before attaching the collection bag to allow the air to equilibrate in the mask and bags were then attached to the 1-way valve and remained there until full (<1 min). Immediately following each sampling period, horses were given their feed allocation as described above.

To calculate the amount of [1-13C] phenylalanine that was converted to 13CO2, it was necessary to determine total CO2 production, which was accomplished by the 6-h primed (18 μmol/kg), constant (12 μmol·kg−1·h−1) i.v. infusion of [13C] sodium bicarbonate on d 9 (22,23). The preparation and administration of the infusion solution and the breath sampling protocols were as described for the [1-13C] phenylalanine infusion. Following the [13C] sodium bicarbonate infusion, catheters were removed and horses were weighed and allocated to a new treatment diet prior to the afternoon (1400 h) feeding. At the end of the d-9 procedures in period 3, horses were returned to the Middleburg Agricultural Research and Extension Center herd.

Sample analysis procedures

**Blood sample collection and storage.** All blood samples were collected into Vacutainers with sodium heparin additive (Becton Dickinson). Samples were immediately centrifuged at 1500 × g for 10 min at 4°C and the plasma layer was removed and stored at −20°C until the time of analysis.

**Amino acids.** The plasma free amino acid concentrations and feed amino acid content were measured using reverse-phase HPLC (3.9- × 300-mm PICO-TAG reverse phase column; Waters) of phenylisothiocyanate derivatives as recently described (24).

**Plasma phenylalanine enrichment.** The isotopic enrichment of a t-butyldimethylsilyl derivative of phenylalanine (m/z) from plasma samples collected on d 8 was measured using GC-MS (25). Derivatized samples were injected (1 μL at 250°C) into a Focus GC system coupled to a Polaris Q ion-trap MS, which were both operated using Xcalibur software (Thermo Scientific). The temperature gradient in the GC column (Supelco Equity-5 column, 30 m × 0.25 mm × 0.25 μm Sigma-Aldrich) was from 100°C to 300°C at a rate of 10°C/min and helium was the mobile phase at a rate of 1 mL/min. The transfer line and MS were maintained at 300°C and 200°C, respectively. The retention time for phenylalanine was ~13.8 min. The phenylalanine ion chromatographs were monitored for ions with a mass charge ratio of 337 and 336 for the labeled and unlabeled phenylalanine, respectively.

**Breath sample analysis.** The ratio of 13CO2:12CO2 in exhaled breath samples was determined on the same day as sample collection using an isotope selective nondispersive infrared absorption analyzer (IRIS-2; Wagner Analysen Technik Vertriebs).

**Calculations**

**Plasma phenylalanine enrichment.** The plasma enrichment of [1-13C] phenylalanine, in molecules percent excess, was calculated using the formula described by Wilson et al. (24).

**Breath CO2 enrichment.** For each breath sample, the δ enrichment values obtained from the nondispersive infrared absorption analyzer were converted to atoms percent excess using the formula provided by Kingdon et al. (27). For the breath samples collected during the [13C] sodium bicarbonate infusion, total CO2 production was calculated based on breath CO2 enrichment using a previously published formula (22,23,27).

**Primed, constant infusion, whole-body phenylalanine kinetics.** The average plasma enrichment at isotopic steady state (plateau) was used to calculate whole-body phenylalanine kinetics. The plateau in phenylalanine enrichment included at least four values and was defined as having a slope not significantly different from 0 (P > 0.05), as determined using linear regression analysis (GraphPad Prism 4 Software).

The plateau enrichment values were then used to calculate the whole-body phenylalanine flux using a well-established formula (28). Flux includes the amount of the amino acid entering the plasma pool through dietary intake (I), de novo synthesis (N), and protein breakdown (B), or leaving the pool through protein synthesis (Z), oxidation (E), or conversion to other metabolites (M):

\[ Q = I + N + B = Z + E + M. \]

The amount of phenylalanine entering the plasma free amino acid pool from dietary intake (I) was estimated by multiplying dietary phenylalanine intake by 0.5 to account for prececal phenylalanine digestibility (29) and the dietary phenylalanine that is extracted during first-pass splanchnic metabolism in other monogastric species (30,31). Because phenylalanine is an indispensable amino acid in horses (1), de novo synthesis of this amino acid does not occur and the phenylalanine that is released into the free amino acid pool as a result of protein breakdown can be estimated by:

\[ B = Q − I. \]

Phenylalanine oxidation was calculated using the equations provided by Hsu et al. (28). Nonoxidative phenylalanine metabolism can be calculated by taking the difference between phenylalanine flux and oxidation and this can be used as an indicator of whole-body phenylalanine use for protein synthesis. The major non-CO2 product of phenylalanine metabolism is tyrosine. In the present study, the only source of dietary tyrosine was the pelleted diet, which all horses received at the same body weight-corrected intake level. If it is assumed that phenylalanine conversion to tyrosine represented only a small amount of total phenylalanine metabolism, and one that was not affected by study diet, then assuming that phenylalanine flux is not affected by diet, any reduction in whole-body phenylalanine oxidation would signify an increase in phenylalanine use for whole-body protein synthesis.

**Statistical analysis**

Unless otherwise stated, all data were analyzed using the mixed procedure of SAS version 9.2 (SAS Institute) and data were considered significant at P < 0.05. Because of the relatively large age range of the horses, age was initially included as a covariate in all statistical models; however, because there was no effect of age (P > 0.05) on any of the variables studied, it was subsequently removed from the statistical models. When the fixed effects were significant (P < 0.05), preplanned comparisons of least squares means were made using the pdiff test. All data are presented as least squares means ± SE.

The plasma amino acid concentrations on d 7 were analyzed using repeated-measures analysis, with treatment, time, and time × treatment interaction as fixed effects and horse nested in treatment as the random effect. For each treatment, the effect of infusion time on isotopic enrichment in the blood and breath 13C enrichment was assessed using a repeated-measures analysis, with time as the fixed effect and horse as the subject. The variance-covariance matrix was chosen for each repeated-measures analysis based on the lowest value for Schwarz’s Bayesian Criterion. The plateau enrichments in the blood and breath samples and the phenylalanine kinetics parameters were analyzed with a 1-way ANOVA, with treatment as the fixed effect and horse nested in treatment as the random effect. For each treatment, the nonoxidative phenylalanine disposal was compared to the phenylalanine release from protein synthesis using a paired t test.

**Results**

One gelding allocated to receive the +Leu and +Lys treatments had to be removed from the study following the control period for reasons unrelated to the study. Therefore, all measurements for the +Leu and +Lys treatments were n = 5. Additionally, the blood sampling catheter was not patent for one horse receiving the +Glu treatment during the [1-13C]phenylalanine infusion, giving an n = 5 for +Glu for the phenylalanine kinetics parameters.
<table>
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<td>Control +Glu +Lys +Leu +Phe</td>
<td>Treatment P Time Treatment × time</td>
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<td>n</td>
<td>12 6 5 6 6 6</td>
<td>12 6 5 5 6 6</td>
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<td>Alanine</td>
<td>189 ± 15ab 165 ± 21ab 159 ± 23ab 146 ± 23ab 125 ± 21ab</td>
<td>197 ± 14 231 ± 20* 244 ± 22* 214 ± 22* 228 ± 20*</td>
<td>NS &lt;0.0001 0.02</td>
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<td>Arginine</td>
<td>60 ± 8ab 69 ± 11ab 92 ± 12ab 69 ± 12ab 69 ± 11ab</td>
<td>75 ± 10a 112 ± 14ab 142 ± 15ab 119 ± 15ab 126 ± 14ab</td>
<td>NS &lt;0.0001 NS</td>
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<tr>
<td>Aspartate</td>
<td>24 ± 6 28 ± 3 14 ± 3 17 ± 3 8 ± 3</td>
<td>28 ± 5 30 ± 7 27 ± 8 37 ± 8 29 ± 7</td>
<td>NS &lt;0.0001 NS</td>
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<tr>
<td>Asparagine</td>
<td>37 ± 4 33 ± 5 40 ± 6 27 ± 6 41 ± 5</td>
<td>47 ± 5a 56 ± 7ab 93 ± 7a 45 ± 7ab 72 ± 7ab</td>
<td>NS &lt;0.0001 NS</td>
</tr>
<tr>
<td>Glutamate</td>
<td>90 ± 10 90 ± 10 51 ± 16 72 ± 16 34 ± 15</td>
<td>76 ± 9 82 ± 13 59 ± 15 68 ± 15 52 ± 13</td>
<td>NS NS NS</td>
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<td>Glutamine</td>
<td>1060 ± 60 880 ± 80 1000 ± 90 950 ± 90 960 ± 80</td>
<td>1110 ± 60 1240 ± 80a 1250 ± 90* 1070 ± 90 1220 ± 80*</td>
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</tr>
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<td>Glicine</td>
<td>583 ± 30 582 ± 42 532 ± 46 499 ± 46 535 ± 42</td>
<td>544 ± 22 645 ± 45 538 ± 50 497 ± 50 576 ± 45</td>
<td>NS NS NS</td>
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<td>Histidine</td>
<td>72 ± 4ab 73 ± 6ab 78 ± 6ab 72 ± 6ab 58 ± 6ab</td>
<td>82 ± 6ab 75 ± 9a 103 ± 10ab 87 ± 10ab 118 ± 9ab</td>
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<td>Isoleucine</td>
<td>36 ± 2ab 41 ± 3 26 ± 3a 31 ± 3ab 31 ± 3ab</td>
<td>43 ± 4ab 51 ± 5ab 39 ± 6ab 52 ± 6ab 56 ± 5ab</td>
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<td>Lecithin</td>
<td>92 ± 6ab 104 ± 8a 131 ± 9a 84 ± 9a 95 ± 8a</td>
<td>117 ± 11ab 117 ± 16a 185 ± 17ab 123 ± 17ab 124 ± 16a</td>
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<tr>
<td>Lysine</td>
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<td>213 ± 15ab 237 ± 21ab 254 ± 22ab 236 ± 22ab 256 ± 21ab</td>
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<td>Methionine</td>
<td>20 ± 1 21 ± 1 16 ± 2 13 ± 2 16 ± 1</td>
<td>23 ± 3 25 ± 4 24 ± 5 21 ± 5 29 ± 4</td>
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<td>Phenyalanine</td>
<td>50 ± 3 48 ± 4ab 52 ± 4ab 39 ± 4a 72 ± 4c</td>
<td>53 ± 3a 54 ± 4a 58 ± 4a 54 ± 4a 121 ± 4ab</td>
<td>&lt;0.0001 &lt;0.0001 &lt;0.0001</td>
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<td>Proline</td>
<td>83 ± 5ab 77 ± 7ab 70 ± 8ab 60 ± 8a 61 ± 7a</td>
<td>101 ± 7 119 ± 11a 122 ± 12a 124 ± 12a 126 ± 11a</td>
<td>NS &lt;0.0001 0.03</td>
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<tr>
<td>Serine</td>
<td>309 ± 14c 278 ± 20b 227 ± 22ab 231 ± 22ab 210 ± 20a</td>
<td>299 ± 17 317 ± 24 281 ± 26a 249 ± 26 283 ± 24a</td>
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<td>Threonine</td>
<td>80 ± 6ac 71 ± 8abc 84 ± 9a 58 ± 9ab 55 ± 8ab</td>
<td>82 ± 6a 94 ± 8ab 113 ± 9ab 84 ± 9ab 110 ± 8ab</td>
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<td>Tryptophan</td>
<td>65 ± 3 67 ± 4 70 ± 4 54 ± 4 62 ± 4</td>
<td>73 ± 4 74 ± 6 79 ± 6 71 ± 6a 80 ± 6b</td>
<td>NS 0.0002 NS</td>
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<tr>
<td>Tyrosine</td>
<td>47 ± 4b 51 ± 5ab 44 ± 6ab 33 ± 5a 63 ± 5a</td>
<td>51 ± 4a 58 ± 5a 62 ± 5ab 51 ± 6ab 99 ± 5ab</td>
<td>&lt;0.0001 &lt;0.0001 0.01</td>
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<td>Valine</td>
<td>157 ± 7a 157 ± 10b 123 ± 11a 134 ± 11ab 146 ± 10ab</td>
<td>162 ± 7ab 173 ± 9ab 140 ± 11b 164 ± 11ab 176 ± 9ab</td>
<td>0.01 0.02 NS</td>
</tr>
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</table>

1 Values are least squares means ± SEM, as determined using a repeated-measures analysis with autoregression (heterogeneous) used as the variance-covariance matrix. Within a time point, means without a common letter differ, P < 0.05.

2 NS, not significant, P > 0.05.

3 Different from corresponding 0 min, P < 0.05. +Glu, glutamate-supplemented diet; +Leu, leucine-supplemented diet; +Lys, lysine-supplemented diet; +Phe, phenylalanine-supplemented diet.
With the exception of glutamate and glycine, the plasma concentrations of all amino acids measured were affected by time \((P < 0.05)\) following the consumption of the meal, with higher plasma concentrations at \(t = 120\) min than at \(t = 0\) min (Table 1). Treatment affected the plasma concentrations of arginine, histidine, isoleucine, leucine, phenylalanine, serine, tyrosine, and valine \((P < 0.05)\) (Table 1). Horses receiving the +Phe treatment had higher concentrations of phenylalanine and tyrosine than those receiving the other treatment diets, whereas the +Leu treatment resulted in higher leucine but lower isoleucine and valine concentrations in the plasma (Table 1). There was a treatment \(\times\) time interaction for the plasma concentrations of alanine, histidine, lysine, phenylalanine, and tyrosine \((P < 0.05)\) (Table 1).

The plasma and breath enrichments of \([1-^{13}\text{C}]\) phenylalanine and \(^{13}\text{CO}_2\), respectively, changed \((P < 0.05)\) during the \([1-^{13}\text{C}]\) phenylalanine and \([1-^{13}\text{C}]\) sodium bicarbonate infusions (Figs. 1–3). Plasma \([1-^{13}\text{C}]\) phenylalanine enrichments reached plateau by \(t = 60\) min for all treatments (see Fig. 1 for control; other treatments not shown). The breath \(^{13}\text{CO}_2\) enrichments during the \([1-^{13}\text{C}]\) phenylalanine infusion reached a plateau by \(t = 210\) min for control (Fig. 2) and \(t = 180, 210, 240,\) and 270 min for +Lys, +Leu, +Glu, and +Phe, respectively (data not shown). During the \([^{13}\text{C}]\) sodium bicarbonate infusion, breath \(^{13}\text{CO}_2\) enrichments reached plateau by 120 min (Fig. 3). There was a significant effect of treatment \((P < 0.05)\) for both the plasma and breath enrichments during the \([1-^{13}\text{C}]\) phenylalanine infusion, with horses receiving the +Phe treatment having the highest enrichments in both cases, with the plasma enrichments greater than the control and +Glu treatments \((P < 0.05)\) and the breath enrichments greater \((P < 0.05)\) than all other treatments (Table 2). Breath \(^{13}\text{CO}_2\) enrichments at plateau were not affected by treatment during the \([^{13}\text{C}]\) sodium bicarbonate infusion (Table 3). For all plasma and breath enrichments, there was a similar degree of variation of the plateau for all treatments (Table 2). There was no effect of treatment diet on whole-body \(^{12}\text{CO}_2\) production. Phenylalanine flux was affected by treatment \((P < 0.05)\), with horses receiving the +Phe treatment having a higher flux than those receiving the control or +Glu treatments (Table 3). Horses receiving the +Phe treatment also had a greater rate of phenylalanine oxidation than the horses receiving any of the other treatment diets \((P < 0.05);\) Table 3). There was no effect of treatment on either phenylalanine release from protein breakdown or nonoxidative phenylalanine disposal (Table 3). The nonoxidative phenylalanine disposal rate was greater than the rate of phenylalanine release from protein breakdown for the control and +Phe treatments \((P < 0.05)\) (Table 3).

**Discussion**

To the best of our knowledge, this study represents the first time that whole-body protein metabolism has been studied in horses using \([1-^{13}\text{C}]\) phenylalanine kinetics. Using the isotope methodology, we were able to estimate rates of whole-body protein synthesis and breakdown in mature horses and demonstrated that there were no increases in whole-body protein synthesis in response to the addition of any of the supplemental amino acids.

**Development of isotope infusion procedures to study protein metabolism in horses.** A primeconstant ratio of 1.5 resulted in a rapid rise in plasma phenylalanine enrichment, with a plateau maintained from 60 min onward. Plasma phenylalanine enrichments were significantly greater at \(t = 30\) min than during the plateau, suggesting an overpriming of the phenylalanine pool, which is consistent with the fact that the ratio used was slightly greater than the optimal ratio of 1.45 (see Supplemental Materials). Although the plasma phenylalanine enrichments reached a rapid plateau, breath \(^{13}\text{CO}_2\) enrichments took much longer to stabilize during the \([1-^{13}\text{C}]\) phenylalanine infusion, taking up to 270 min to reach a plateau in the horses receiving the +Phe treatment, where phenylalanine oxidation to \(^{12}\text{CO}_2\) was also the greatest. This delay in breath \(^{13}\text{CO}_2\) plateau is attributable to the large \(^{12}\text{CO}_2\) and bicarbonate pool with which the labeled \(^{12}\text{CO}_2\) produced during phenylalanine catabolism must equilibrate (22, 32). To reduce the time to reach breath \(^{13}\text{CO}_2\) isotopic plateau during \([1-^{13}\text{C}]\) phenylalanine infusion, one solution is to administer a priming dose of \([^{13}\text{C}]\) sodium bicarbonate at the same time as the priming dose of \([1-^{13}\text{C}]\) phenylalanine, as has been commonly done in human studies (33). Alternatively, if total \(^{12}\text{CO}_2\) production is measured using \([^{13}\text{C}]\) sodium bicarbonate (23) immediately prior to the \([1-^{13}\text{C}]\) phenylalanine infusion, this would serve the additional purpose of priming the bicarbonate pool and is expected to expedite the attainment of breath \(^{13}\text{CO}_2\) plateau (34), such that both infusions could be conducted within an ~6-h period. Measuring total \(^{12}\text{CO}_2\) production and phenylalanine kinetics on the same day will offer a substantial improvement over the methods used in the current study.

One of the objectives of this research was to develop isotope infusion methodologies that could subsequently be used in IAAO studies to determine amino acid requirements in horses. For IAAO studies using phenylalanine as the indicator, it is essential that phenylalanine and tyrosine intakes exceed the requirements (4). Phenylalanine and tyrosine requirements have not been established in horses (1). Using the lysine requirement of mature horses (1) in combination with the phenylalanine and tyrosine content, relative to lysine content, of equine skeletal muscle (35), the phenylalanine (28 mg·kg\(^{-1}\)·d\(^{-1}\)) and tyrosine (23 mg·kg\(^{-1}\)·d\(^{-1}\)) requirements of mature horses can be estimated. The control diet provided 54 mg·kg\(^{-1}\)·d\(^{-1}\) phenylalanine and 38 mg·kg\(^{-1}\)·d\(^{-1}\) tyrosine (Supplemental Table 2) and therefore appears to provide ample amounts of both amino acids. The elevated plasma tyrosine concentrations in horses receiving the +Phe treatment is likely due to the fact that the supplemental phenylalanine intake resulted in a total aromatic amino acid intake (155 mg·kg\(^{-1}\)·d\(^{-1}\)) that exceeded the oxidative capacity for these amino acids, resulting in an accumulation of tyrosine in the body. This was previously

![FIGURE 1](https://academic.oup.com/jn/article-abstract/142/3/461/4743470)

**FIGURE 1** Plasma \([1-^{13}\text{C}]\) phenylalanine enrichment in horses receiving the control treatment during the \([1-^{13}\text{C}]\) phenylalanine infusion. Values are mean ± pooled SEM, \(n = 12\), as determined using repeated-measures analysis with the compound symmetry variance-covariance matrix. Means without a common letter differ, \(P < 0.05\).
described in neonatal piglets (36) and it was noted that phenylalanine oxidation to CO₂ was not linear when phenylalanine intakes exceeded a certain threshold. In future IAAO studies in horses, it will be essential to ensure not only that adequate aromatic amino acids are provided in the diet but also that the levels of intake are not too high, because this would impair the ability of phenylalanine oxidation to respond to differences in test amino acid intake. Additional research is needed to determine optimal levels of aromatic amino acid intake.

To determine amino acid requirements using the IAAO methodology, it is also important that phenylalanine intake is equal between treatment groups, so that reductions in phenylalanine oxidation are indicative of increases in phenylalanine utilization for whole-body protein synthesis. In the case of the +Phe treatment in the present study, this condition was not met and therefore caution must be used when interpreting the oxidation data from the +Phe group. Horses receiving the +Phe treatment had the highest rates of phenylalanine oxidation, which based on IAAO methodology would indicate lower rates of whole-body protein synthesis compared to the other treatments. However, whole-body protein synthesis was not lower for the +Phe treatment, because the supplemental phenylalanine caused an expansion of the phenylalanine pool and increased phenylalanine flux. In humans and piglets, as phenylalanine intake increased above the requirement, there were increases in whole-body protein synthesis compared to the other treatments.

According to the IAAO methodology (7), if one of the supplemented amino acids had been limiting to whole-body protein synthesis in the control diet, then the addition of this amino acid to the control diet would have decreased phenylalanine oxidation (7). However, despite the fact that the supplemental amount of leucine and lysine appear to have been absorbed by the horses, there were no changes in phenylalanine oxidation in response to lysine or leucine supplementation; therefore, protein synthesis was not limited by the amounts of these amino acids provided in the control diet. This is confirmed by similar rates of nonoxidative phenylalanine disposal for all treatment groups.

Although it is possible that an amino acid other than one of the those supplemented was the first limiting amino acid in the control diet, this is unlikely for several reasons. First, although oil was added to the diet to provide sufficient DE to maintain the body weight of the mature Arabian geldings while minimizing the degree to which crude protein was in excess of the estimated requirements, crude protein was still provided at ~135% of the current requirement (1), resulting in a considerable excess of total amino nitrogen and explaining the lack of effect of glutamate supplementation. Secondly, lysine and threonine have been proposed as the first and second limiting amino acids, respectively, in typical horse diets (10,40). The control diet provided lysine at ~2 times the current requirement for mature horses and threonine at a level of 43 mg·kg⁻¹·d⁻¹ (~21.5 g/d for a 500-kg horse) (Supplemental Table 2). Although there is currently no threonine requirement provided for horses of any age, the NRC (1) suggests the threonine requirement can be approximated by multiplying the ratio of threonine:lysine in skeletal muscle by the lysine requirement, which gives an
estimated threonine requirement of 16.3 g/d for a 500-kg mature horse (1). Therefore, the control diet should have provided sufficient threonine, although to confirm this, a similar study could be conducted in which the control diet is supplemented with threonine. Our data support that the control diet provided adequate amounts of the indispensable amino acids to support maximum rates of whole-body protein synthesis in mature horses at rest.

**Whole-body protein metabolism in mature horses.** Although the present study did not provide evidence for a limiting amino acid in the control diet, it did provide valuable information regarding whole-body protein metabolism in horses. Regardless of diet, the values for phenylalanine release from protein breakdown and nonoxidative phenylalanine disposal are numerically similar, although the differences were significant for the control and +Phe diets. Because nonoxidative phenylalanine disposal includes both phenylalanine use for protein synthesis and phenylalanine that is converted to tyrosine (41), the differences between the nonoxidative phenylalanine disposal and the protein breakdown rates can likely be accounted for by tyrosine synthesis. In humans, the amount of phenylalanine converted to tyrosine is very small compared to the amount of phenylalanine used for protein synthesis (42) and humans consuming >7 mg·kg⁻¹·d⁻¹ of tyrosine formed only ~2 μmol·kg⁻¹·h⁻¹ tyrosine from phenylalanine (43), consistent with the differences between the nonoxidative phenylalanine disposal and protein breakdown rates measured in the Arabian horses. These findings indicate that rates of protein synthesis and breakdown were equal in the Arabian horses, that there was no net protein accretion in these horses, regardless of diet, and supports that the individual indispensable amino acid requirements were met by the control diet. The horses in this study were mature, weight-stable horses and were not expected to be actively accreting protein, and this is clearly supported by the isotopic data. The majority of nitrogen retention studies in horses have been performed in growing (10) or exercising (44) horses; however, a recent study in lightly exercised mature horses found that horses receiving a hay and grain diet had a slightly positive nitrogen balance (5.4 g/d for ~500-kg horses), indicating a small amount of daily protein accretion (45). The differences between the previous (45) and present studies may be attributable to differences in activity level or because the previous study did not account for cutaneous nitrogenous losses in their nitrogen balance calculations, which have been estimated at ~4 g/d for a 500-kg horse (46). Neither body

### TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+Glu</th>
<th>+Lys</th>
<th>+Leu</th>
<th>+Phe</th>
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<td>5</td>
<td>5</td>
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<tr>
<td>Plasma [1,13C] phenylalanine MPE</td>
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<tr>
<td>MPE</td>
<td>12.2 ± 0.4ab</td>
<td>12.6 ± 0.6b</td>
<td>12.0 ± 0.6ab</td>
<td>11.4 ± 0.6ab</td>
<td>10.2 ± 0.5ab</td>
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<tr>
<td>CV, %</td>
<td>6.6 ± 1.3</td>
<td>5.9 ± 1.8</td>
<td>7.4 ± 1.8</td>
<td>9.7 ± 1.8</td>
<td>11.8 ± 1.8</td>
<td>NS²</td>
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<tr>
<td>Breath [13CO₂ APE during [1,13C] phenylalanine infusion</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APE</td>
<td>0.008 ± 0.001a</td>
<td>0.008 ± 0.001a</td>
<td>0.009 ± 0.001a</td>
<td>0.009 ± 0.001a</td>
<td>0.012 ± 0.001b</td>
<td>0.002</td>
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<tr>
<td>CV, %</td>
<td>12.1 ± 1.6</td>
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<td>11.3 ± 2.3</td>
<td>14.6 ± 2.3</td>
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<td>Breath [13CO₂ APE during [13C] sodium bicarbonate infusion</td>
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<td>APE</td>
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<td>0.105 ± 0.004</td>
<td>0.104 ± 0.004</td>
<td>0.106 ± 0.004</td>
<td>0.104 ± 0.004</td>
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<tr>
<td>CV, %</td>
<td>2.4 ± 0.3</td>
<td>2.2 ± 0.4</td>
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<td>2.9 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹ Values are least squares means ± SEM. Labeled means in a row without a common letter differ, P < 0.05. APE, atoms percent excess; +Glu, glutamate-supplemented diet; +Leu, leucine-supplemented diet; +Lys, lysine-supplemented diet; +Phe, phenylalanine-supplemented diet. ² NS, not significant, P > 0.05.

### TABLE 3

<table>
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<tr>
<th></th>
<th>Control</th>
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<th>+Lys</th>
<th>+Leu</th>
<th>+Phe</th>
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<td>Phenylalanine flux, μmol · kg⁻¹ · h⁻¹</td>
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<tr>
<td></td>
<td>41.1 ± 1.6ab</td>
<td>39.2 ± 2.4ab</td>
<td>44.2 ± 2.4ab</td>
<td>41.7 ± 2.4ab</td>
<td>49.2 ± 2.2ab</td>
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<td>Carbon dioxide production, mmol · kg⁻¹ · h⁻¹</td>
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<td></td>
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<tr>
<td></td>
<td>10.3 ± 0.2</td>
<td>10.9 ± 0.3</td>
<td>10.9 ± 0.3</td>
<td>11.1 ± 0.3</td>
<td>11.0 ± 0.3</td>
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<td>Phenylalanine entering the free phenylalanine pool</td>
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<tr>
<td>Phenylalanine from dietary intake, μmol · kg⁻¹ · h⁻¹</td>
<td>7.7 ± 0.0a</td>
<td>7.7 ± 0.0a</td>
<td>7.7 ± 0.0a</td>
<td>7.7 ± 0.0a</td>
<td>15.5 ± 0.0b</td>
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<tr>
<td>Phenylalanine from protein breakdown, μmol · kg⁻¹ · h⁻¹</td>
<td>33.4 ± 1.6</td>
<td>31.6 ± 2.4</td>
<td>36.7 ± 2.4</td>
<td>34.0 ± 2.4</td>
<td>33.7 ± 2.2</td>
<td>NS</td>
</tr>
<tr>
<td>Phenylalanine leaving the free phenylalanine pool</td>
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</tr>
<tr>
<td>Phenylalanine oxidation, μmol · kg⁻¹ · h⁻¹</td>
<td>5.7 ± 0.5a</td>
<td>6.3 ± 0.8a</td>
<td>7.2 ± 0.8a</td>
<td>7.2 ± 0.8a</td>
<td>12.0 ± 0.7a</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nonoxidative phenylalanine disposal, μmol · kg⁻¹ · h⁻¹</td>
<td>39.4 ± 1.4a</td>
<td>32.9 ± 2.0a</td>
<td>37.2 ± 2.0a</td>
<td>34.5 ± 2.0a</td>
<td>37.2 ± 1.8a</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹ Values are least squares means ± SEM. Labeled means in a row without a common letter differ, P < 0.05. *Differs from the rate of nonoxidative phenylalanine disposal for that treatment, P < 0.05. +Glu, glutamate-supplemented diet; +Leu, leucine-supplemented diet; +Lys, lysine-supplemented diet; +Phe, phenylalanine-supplemented diet.
² The following stochastic model of phenylalanine kinetics was used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal.
³ NS, not significant, P > 0.05.
composition nor body weight changed throughout the previous study (45) and so the latter seems to be the most plausible explanation for the differences between the isotopic and nitrogen balance data regarding protein accretion in mature horses.

To summarize, the isotope infusion methodologies resulted in the rapid attainment of isotopic plateau in blood [1-13C] phenylalanine enrichments, with a slower rise to plateau in the breath 13CO2 enrichments. The rate of whole-body protein synthesis supported by the control diet was not increased by the addition of any of the supplemental amino acids, indicating that none of these amino acids were limiting to protein synthesis in the control diet. Furthermore, the isotope kinetics data showed that mature horses receiving crude protein in excess of the NRC requirements (1) are in a state of whole-body protein balance. The methods developed in this study represent a valuable first step in understanding whole-body protein metabolism in mature horses and with minor refinements will be useful in future investigations of amino acid requirements and metabolism in horses of a variety of ages and physiological states.

Acknowledgments
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