Stable isotope studies reveal pathways for the incorporation of non-essential amino acids in Acyrthosiphon pisum (pea aphids)

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ABSTRACT
Plant roots incorporate inorganic nitrogen into the amino acids glutamine, glutamic acid, asparagine and aspartic acid, which together serve as the primary metabolites of nitrogen transfer to other tissues. Given the preponderance of these four amino acids, phloem sap is a nutritionally unbalanced diet for phloem-feeding insects. Therefore, aphids and other phloem feeders typically rely on microbial symbionts for the synthesis of essential amino acids. To investigate the metabolism of the four main transport amino acids by the pea aphid (Acyrthosiphon pisum), and its Buchnera aphidicola endosymbionts, aphids were fed defined diets with stable isotope-labeled glutamine, glutamic acid, asparagine or aspartic acid (U-13C, U-15N; U-13C; α-15N; or γ-15N). The metabolic fate of the dietary 15N and 13C was traced using gas chromatography–mass spectrometry (GC-MS). Nitrogen was the major contributor to the observed amino acid isotopomers with one additional unit mass (M+1). However, there was differential incorporation, with the amine nitrogen of asparagine being incorporated into other amino acids more efficiently than the amide nitrogen. Higher isotopomers (M+2, M+3 and M+4) indicated the incorporation of varying numbers of 13C atoms into essential amino acids. GC-MS assays also showed that, even with an excess of dietary labeled glutamine, glutamic acid, asparagine or aspartic acid, the overall content of these amino acids in aphid bodies was mostly the product of catabolism of dietary amino acids and subsequent re-synthesis within the aphids. Thus, these predominant dietary amino acids are not passed directly to Buchnera endosymbionts for synthesis of essential amino acids, but are rather are produced de novo, most likely by endogenous aphid enzymes.

KEY WORDS: Buchnera aphidicola, Aphid, Endosymbiont, Glutamic acid, Glutamine, Aspartic acid, Asparagine, Biosynthesis, Unbalanced diet

INTRODUCTION
The majority of inorganic nitrogen assimilation in plants occurs in the roots via the glutamine synthase–glutamate oxoglutarate aminotransferase metabolic cycle (Coruzzi and Last, 2000; Oaks, 1992). Aspartic acid aminotransferase and asparagine synthase use glutamic acid and glutamine, respectively, as nitrogen donors to produce aspartic acid and asparagine. Assimilation of inorganic nitrogen sources varies somewhat among different plant species (Fischer et al., 1998; Okumoto and Pilot, 2011; Oliveira et al., 2013). Nevertheless, the four amino acids of primary nitrogen assimilation, and less commonly also serine and/or alanine, constitute more than half of all transported nitrogen in most plant species (Douglas, 1993; Febvay et al., 1988; Fukumori and Chino, 1982; Girousse and Bournoville, 1994; Girousse et al., 1996; Hunt et al., 2006; Karley et al., 2002; Lohaus and Moellers, 2000; Ponder et al., 2000; Riens et al., 1991; Sandström and Pettersson, 1994; Sasaki et al., 1990; Urquhart and Joy, 1981; Valle et al., 1998; Wilkinson and Douglas, 2003). Whereas these four non-essential amino acids are relatively abundant, phloem plant sap typically is deficient in essential amino acids that cannot be synthesized by animals (Atkins et al., 2011; Girousse et al., 2005; Lohaus et al., 1994; Zhang et al., 2010).

Animals that feed on nutritionally unbalanced resources such as phloem sap, which typically has sub-optimal amounts of essential amino acids, have evolved mechanisms for acquiring or synthesizing these amino acids. In many cases, this involves symbiotic interactions with microorganisms that produce essential amino acids whose synthesis is not encoded in animal genomes (Cotti et al., 2010; Defossez et al., 2011; Nardi et al., 2002; Oh et al., 2010; Russell et al., 2009; Schloss et al., 2006; Spitteler et al., 2000). Some of the best-studied symbioses of this type are found in aphids, whiteflies and other phloem-feeding Hemiptera (Braendle et al., 2003; Douglas et al., 2006; Kikuchi et al., 2007, 2009; Wilkinson et al., 2001).

Phloem-feeding insects benefit from having evolved rapid and efficient pathways for the uptake and metabolic conversion of glutamine, glutamic acid, asparagine and aspartic acid into essential amino acids. Aphids accomplish this in part through close interactions with endosymbiont bacteria, Buchnera aphidicola, that are contained in specialized cells called bacteriocytes (Buchner, 1965). Although all Buchnera synthesize essential nutrients for their aphid hosts, there is variation among different aphid species and even lineages within a species in the metabolic functions of their bacterial endosymbionts and their utilization of host plant nutrients (MacDonald et al., 2011; Vogel and Moran, 2011). In addition to Buchnera, several aphid species have been shown to contain facultative endosymbionts that can contribute to survival and fitness (Leonardo and Muiru, 2003; Oliver et al., 2003), including through amino acid biosynthesis (Richards et al., 2010).

Sequencing of the pea aphid (Acyrhysphon pisum) and Buchnera genomes, gene expression profiling, and proteomic studies have provided new insight into the predicted pathways of amino acid biosynthesis in the A. pisum–Buchnera symbiosis (Brinza et al., 2010; Hansen and Moran, 2011; Poliakov et al., 2011; Richards et al., 2010; Shigenobu et al., 2000). Notably, there are several examples of shared biosynthesis pathways for essential amino acids that are partly encoded by the endosymbiont bacteria and partly by the aphid host (Russell et al., 2013; Wilson et al., 2010). Genomic analyses indicate that aspartic acid and glutamic acid contribute to the biosynthesis of all amino acids in the aphid–Buchnera system by providing nitrogen and/or the carbon backbone (Hansen and Moran, 2011).

The de novo synthesis of essential amino acids from other phloem nitrogen sources has been explored in the symbiosis between
A. pisum and its Buchnera endosymbiont. Analysis of A. pisum fed on Vicia faba plants and artificial diet containing varying amounts of non-essential and essential amino acids showed a similar overall amino acid composition, suggesting biosynthesis of essential amino acids within the insects (Liadouze et al., 1995). Compared with aphids containing Buchnera, aposymbiotic aphids that had been cleared of endosymbionts by antibiotic treatment accumulated greater amounts of free amino acids when feeding from plants (Liadouze et al., 1995). Whereas asparagine, aspartic acid and glutamine were more abundant, several essential amino acids were less abundant, suggesting that the endosymbiont bacteria contribute to the conversion of non-essential amino acids into essential amino acids.

Experiments with isolated bacteriocytes showed that they actively take up [U-14C]glutamine and convert it into glutamic acid, which is efficiently taken up by Buchnera bacteria (Sasaki and Ishikawa, 1995). In whole-aphid experiments, de novo synthesis of essential amino acids in A. pisum was demonstrated by incorporation of carbon from [13C]sucrose in the diet (Febvay et al., 1999). The relative incorporation of 14C from labeled amino acids and sucrose was markedly different in A. pisum feeding on a diet containing excess amino acids compared with those on a diet that was designed to mimic host phloem sap components, indicating that aphids and/or their symbionts adjust amino acid synthesis based on their dietary needs (Febvay et al., 1999; Liadouze et al., 1995).

Although 14C labeling experiments are very sensitive for detecting dietary carbon incorporation into essential amino acids, measurement of the radioactive signal provides only partial information about metabolic pathways. In particular, 14C incorporation by itself cannot determine whether intact dietary amino acids are provided to the Buchnera endosymbionts for the synthesis of essential amino acids. In contrast, mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) techniques, in conjunction with labeled non-radioactive stable isotopes such as 13C and 15N, permit the detection of specific labeled atoms in the reaction products (Bier, 1997; Crown and Antoniewicz, 2013; Kahana et al., 1998; Pond et al., 2006; Schatschneider et al., 2011; Tsvetanova et al., 2002; Venema et al., 2007). For instance, stable isotope labeling has been used to elucidate pathways in metabolic engineering studies (Alonso et al., 2007; Chen et al., 2011; Schwender et al., 2003; Suthers et al., 2007).

The MS fragmentation pattern and the elemental composition of the fragments can be used to trace the origin of the carbon and nitrogen incorporation from dietary non-essential amino acids into essential amino acids in the aphid–Buchnera system. Stable isotope labeling experiments with [13C]glutamine provided insight into aphid nitrogen incorporation (Sasaki et al., 1990; Sasaki and Ishikawa, 1995). When [8,15N]glutamine was added to bacteriocytes, the 15N was incorporated into glutamic acid, indicating likely de novo synthesis from α-ketoglutarate. Isolated Buchnera actively took up labeled [15N] glutamic acid and incorporated the labeled nitrogen into other amino acids. Together, these experiments suggested that amide (δ) nitrogen and amine (α) nitrogen of glutamine are utilized differently in the aphid–Buchnera system.

Here, we describe experiments in which we used gas chromatography–mass spectrometry (GC-MS) to determine the metabolic fates of both carbon and nitrogen derived from the main phloem transport amino acids after they are taken up by whole aphids. Specifically, we set out to answer the following questions in the A. pisum–Buchnera metabolic system. (i) Does utilization of the four predominant nitrogen transport amino acids, aspartic acid, asparagine, glutamic acid and glutamine, follow the predicted metabolic pathways? (ii) Are the two nitrogen atoms in asparagine functionally equivalent? (iii) Is the carbon backbone of dietary amino acids maintained during the synthesis of essential amino acids?

**MATERIALS AND METHODS**

**Aphid rearing**

A clonal culture of the pea aphid (Acyrthosiphon pisum) strain CWR09/18, derived from a single female collected in June 2009 from an alfalfa crop at Freeville Farms (Freeville, NY, USA) (Russell et al., 2014), was reared on fava beans (Vicia faba, var. Windsor; Johnny’s Selected Seeds, Winslow, ME, USA), which were grown in Metromix 200 (Scotts, Marysville, OH, USA) in a growth chamber at 23°C and 100 μmol photons m−2 s−1 light intensity, with a 18 h:6 h light:dark cycle; 10 day old pre-flowering plants were used for aphid rearing.

**Chemicals and reagents**

All labeled amino acids were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). N-Methyl-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich (St Louis, MO, USA). Reagent grade hydrochloric acid was purchased from VWR (Rudnor, PA, USA).

**Aphid diets**

To study the specific uptake and incorporation of phloem transport amino acids, aphids were fed with artificial diet prepared as described by Prosser and Douglas (1991), with modifications (A. E. Douglas, personal communication) (Table S1). The diets for labeling experiments were modified so that they contained combinations of either aspartic acid and asparagine, or glutamic acid and glutamine, as the only nitrogen sources. To achieve the required pH 7 in the diet, aspartic acid and asparagine were added in an approximately 1:3.5 ratio (33.2 mmol l−1 aspartic acid and 115.1 mmol l−1 asparagine). Experimental diets were prepared with combinations of isotope-labeled and unlabeled aspartic acid and asparagine. Similarly, diets were prepared with isotope-labeled and unlabeled glutamic acid and glutamine in an approximately 1:2 ratio (50.4 mmol l−1 glutamic acid and 99.0 mmol l−1 glutamine). Acyrthosiphon pisum did not survive for the duration of the experiments when other buffers were used to adjust the pH to 7 in diets containing only aspartic acid or glutamic acid as the nitrogen source.

Baseline values for m/z of fragment ions for all detectable unlabeled amino acids in aphids were obtained using a diet of unlabeled aspartic acid+asparagine to obtain the natural isotopic abundance values. To test the incorporation of carbon and nitrogen into amino acids, experiments were conducted with one of the tested amino acids universally labeled with13C and 15N. These diets consisted of: (i) [U-15N]asparagine; (ii) aspartic acid+[1,15N]asparagine; (iii) [1,15N]glutamic acid+glutamine; and (iv) glutamic acid+[1,15N]glutamine. To determine nitrogen incorporation, two combinations of diets were used: (i) aspartic acid+[1,15N]asparagine; and (ii) glutamic acid+[1,15N]glutamine. To investigate differential nitrogen uptake of the α- and γ-nitrogen of asparagine, aphid feeding experiments were conducted with: (i) aspartic acid+[α-15N]asparagine; (ii) aspartic acid+[γ-15N]asparagine; and (iii) aspartic acid+[1-15N]asparagine. The percentage of isotope-labeled nitrogen in each aphid diet is shown in Table 1.

**Table 1. Percentage of isotope-labeled nitrogen in different aphid diets**

<table>
<thead>
<tr>
<th>Amino acids in the diet</th>
<th>% 15N</th>
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<tbody>
<tr>
<td>50.4 mmol l−1 glutamic acid+99 mmol l−1 glutamine</td>
<td>0</td>
</tr>
<tr>
<td>50.4 mmol l−1 [U-13C, 15N]glutamic acid+99 mmol l−1 glutamine</td>
<td>20</td>
</tr>
<tr>
<td>50.4 mmol l−1 glutamic acid+99 mmol l−1 [U-13C, U-15N]glutamine</td>
<td>80</td>
</tr>
<tr>
<td>50.4 mmol l−1 glutamic acid+99 mmol l−1 [U-13C, U-15N]glutamine</td>
<td>80</td>
</tr>
<tr>
<td>33 mmol l−1 aspartic acid+115 mmol l−1 asparagine</td>
<td>0</td>
</tr>
<tr>
<td>33 mmol l−1 [U-13C, 15N]aspartic acid+115 mmol l−1 asparagine</td>
<td>13</td>
</tr>
<tr>
<td>33 mmol l−1 aspartic acid+115 mmol l−1 [U-13C, U-15N]asparagine</td>
<td>87</td>
</tr>
<tr>
<td>33 mmol l−1 aspartic acid+115 mmol l−1 [U-13C, U-15N]asparagine</td>
<td>87</td>
</tr>
<tr>
<td>33 mmol l−1 aspartic acid+115 mmol l−1 [γ-15N]asparagine</td>
<td>44</td>
</tr>
<tr>
<td>33 mmol l−1 aspartic acid+115 mmol l−1 [γ-15N]asparagine</td>
<td>44</td>
</tr>
</tbody>
</table>
Aphid bioassays
Experiments were conducted with 2 day old larvae that had been deposited by apterous adults onto *V. faba*. Twenty to 30 individual larvae were transferred to 3 cm wide, 10 ml plastic cups that were covered with fine polypropylene mesh at the bottom. Then, the cups were covered with thinly stretched Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA). A 100 µl sample of the diets to be tested was pipetted onto the center of the Parafilm and covered with a second layer of stretched Parafilm. Cups were transferred to an air tight 60×30×30 cm Plexiglas box with a 250 ml beaker containing saturated salt solution to maintain humidity. The box was placed at 22–23°C, 25 µmol photons m⁻² s⁻¹ light intensity, with a 18 h:6 h light:dark cycle. Aphids were harvested for GC-MS analysis of metabolites after 5 days.

Acid hydrolysis for extraction of total amino acids
A weighed amount of aphids from each diet cage was transferred to a 2 ml glass vial. After addition of 400 µl of 6 mol l⁻¹ HCl, the vials were flushed with nitrogen for 30 s, and immediately capped. Aphids were extracted for 36–40 h in a heating block maintained at 110°C. The reaction mixture was cooled and the samples were filtered using 42 µm, 500 µl microcentrifuge filters (Millipore, Billerica, MA, USA). The clear extracts were then evaporated to dryness in a 2 ml glass vial under a nitrogen stream at 60–65°C. The residue was re-dissolved in 10 µl of 20 mmol l⁻¹ HCl, the vials were flushed with nitrogen and the temperature gradient was 115°C for 1.5 h. The samples were then directly injected into a VF-17ms FS, 30 m×0.25 µm FactorFour GC column (Varian) for chromatography. The flow rate was 1 ml min⁻¹, and the temperature gradient was 70°C for 2 min; to 100°C at 5°C min⁻¹, then directly injected into a VF-17ms FS, 30 m×0.25 µm FactorFour GC column (Varian) for chromatography. The flow rate was 1 ml min⁻¹, and the temperature gradient was 70°C for 2 min; to 100°C at 5°C min⁻¹, then held for 5 min; to 150°C at 5°C min⁻¹, held for 0 min; and to 300°C at 12°C min⁻¹ and held for 3 min. Electron ionization (EI) MS spectra were collected at 70 eV. The mass of individual chromatographic peaks was compared with a spectral library (Palisade Corporation, Ithaca, NY, USA), as well as with the retention times and mass spectra of isotope-labeled and unlabeled amino acid standards. Chemical ionization (CI)-MS data, using methane as the ionizing gas, were collected with GC parameters at 70 eV, as described above for EI-MS, and the spectral data were used in assessing parent ions of components that were labeled with more than one atom. Table 2 shows the fragment ions that were monitored to identify and measure the ion abundance of the amino acids seen in the mass spectra.

Table 2. Ions monitored for different metabolite GC retention times

<table>
<thead>
<tr>
<th>Metabolites measured</th>
<th>Retention time (min)</th>
<th>Base peak in EI mode</th>
<th>[Molecular ion +H⁺] in CI mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>6.614</td>
<td>116</td>
<td>234 (2 TMS)</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.074</td>
<td>102</td>
<td>220 (2 TMS)</td>
</tr>
<tr>
<td>Valine</td>
<td>8.922</td>
<td>144</td>
<td>262 (2 TMS)</td>
</tr>
<tr>
<td>Leucine</td>
<td>11.018</td>
<td>158</td>
<td>276 (2 TMS)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>11.839</td>
<td>158</td>
<td>276 (2 TMS)</td>
</tr>
<tr>
<td>Proline</td>
<td>14.967</td>
<td>142</td>
<td>260 (2 TMS)</td>
</tr>
<tr>
<td>Serine</td>
<td>15.126</td>
<td>218</td>
<td>322 (3 TMS)</td>
</tr>
<tr>
<td>Threonine</td>
<td>15.475</td>
<td>218</td>
<td>336 (3 TMS)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>21.487</td>
<td>232</td>
<td>350 (3 TMS)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>24.097</td>
<td>246</td>
<td>364 (3 TMS)</td>
</tr>
<tr>
<td>Pyroglutamic acid</td>
<td>24.317</td>
<td>156</td>
<td>274 (2 TMS)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>24.917</td>
<td>192</td>
<td>310 (2 TMS)</td>
</tr>
<tr>
<td>Lysine</td>
<td>27.042</td>
<td>174</td>
<td>291 (2 TMS)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>28.718</td>
<td>179</td>
<td>398 (3 TMS)</td>
</tr>
</tbody>
</table>

EI, electron ionization; CI, chemical ionization; TMS, trimethylsilyl group.

Data analysis
The total ion abundances for selected ion peaks were calculated by integrating the area under each component and were converted to a percentage proportion of the peak area of the natural isotope mass species [M⁺] (carbon 12, hydrogen 1, sulfur 32, silicone 28 and oxygen 16). In addition to molecular species, less abundant natural isotopes produce [M+1⁻¹] and [M+2⁻²], depending on the molecular composition of the intact compound. This could be observed best in CI-MS. In EI-MS mode, we could measure the fragment ion mass of the base peak [M-117, M-COOTMS], with loss of the trimethylsilyl (TMS)-derivatized carboxyl group, hereafter referred to as M through the entire paper. If one or more 13C- or 15N-labeled atoms are incorporated into the molecule, the intensity of [FM+1⁻¹] would be much higher than the natural abundance. This is illustrated in Fig. 1 and Fig. S1.

Statistical analyses (one-way ANOVA) were conducted to identify significant differences between different experiments using JMP 9 and 11 (SAS Statistical Software, Cary, NC, USA) or Statistix 8 (Analytical Software, Tallahassee, FL, USA). Mean values and standard errors for graphs were calculated using one of the above software packages or Excel (Microsoft, Redmond, WA, USA).

RESULTS
Incorporation of nitrogen from phloem nitrogen transport amino acids
To investigate the incorporation of dietary glutamine, glutamic acid, asparagine and aspartic acid, aphids were raised for 5 days on diets containing these amino acids with 13C and 15N stable isotope labeling. During the course of these experiments, the mass of the aphids increased approximately threefold, indicating uptake and incorporation of nutrients from the artificial diet (Fig. S2).

Incorporation of both the carbon and the nitrogen atoms of dietary amino acids into other amino acids can be detected by GC-MS. As an example, Fig. 1 shows scenarios for the conversion of [U-13C, U-15N]asparagine into isoleucine in the *A. pisum*–Buchnera system. The predicted pathway for isoleucine biosynthesis from asparagine includes both aphid- and Buchnera-encoded enzymes (Hansen and Moran, 2011; Russell et al., 2013). If four carbons of [U-13C, U-15N]asparagine are incorporated into isoleucine, this would result in an M+4 isotopomer (Fig. 1A). The nitrogen atoms of [U-13C, U-15N]asparagine would be lost in the isoleucine biosynthesis pathway, but 15N could be added back during the final transamination step, resulting in an M+5 isotopomer, or instead an M+1 isotopomer if the carbon backbone of asparagine is not conserved. For aphids on diets with [U-13C, U-15N]asparagine, both the M+5 and the M+1 isomers of isoleucine were detected. For aphids on diets with [U-15N]asparagine, the M+1 isomer became more abundant than for those on diets with unlabeled asparagine (Fig. 1C–E), indicating that this M+1 mass increase primarily results from dietary 15N, rather than incorporation of a single 13C atom.

In further experiments, diets containing each of the four [U-13C, U-15N]-labeled amino acids (aspartate, asparagine, glutamate and glutamine) resulted in a mass unit increase in m/z [FM+1] in the [M-117, M-COOTMS] fragment ion (after loss of the TMS-derivatized carboxyl group) in most of detected amino acids (Fig. 2). This indicated that 15N or 13C from the labeled amino acids was incorporated into the newly formed amino acids. However, it is more likely that nitrogen is incorporated into the molecule, as the detected ion fragment retains the amino group from the corresponding amino acids (M-117, shown for isoleucine in Fig. 1E). In the case of lysine, a smaller amount of the FM+2 molecular ion was observed (data not shown), likely as a result of the less frequent incorporation of 15N into both nitrogen atoms of this amino acid.

The incorporation of nitrogen from the [U-13C, U-15N]asparagine and [U-13C, U-15N]glutamine diets was similar for all of the
detected amino acids (Fig. 2). After 5 days on the artificial diet, up to 50% of the individual aphid amino acids were labeled with a single $^{15}$N or $^{13}$C. Nitrogen or carbon incorporation into isoleucine was significantly higher with $[U-^{13}$C, $U-^{15}$N]asparagine relative to $[U-^{13}$C, $U-^{15}$N]glutamine in the diet. In contrast, labeled alanine, glycine, serine and valine were more abundant in aphids fed with $[U-^{13}$C, $U-^{15}$N]glutamine diet (Fig. 2).

In the experiments with labeled aspartic acid or glutamic acid, the observed incorporation of $^{15}$N or $^{13}$C into aphids was much lower than for asparagine and glutamine (Fig. 2). This is largely as a result of limitations of the experimental system. Whereas aspartic acid and glutamic acid each have one nitrogen that can be labeled, asparagine and glutamine have two. Moreover, to achieve the required pH 7 in the diet, it was necessary to add larger amounts of asparagine and glutamine, respectively, showing incorporation of one $^{15}$N atom in electron ionization (EI) mode. Insets show an expansion of the area containing the molecular ion peaks of the labeled isomers. (C,D) Mass spectra of unlabeled and labeled isoleucine produced from $[U-^{13}$C, $U-^{15}$N]asparagine, respectively, showing incorporation of one $^{15}$N atom in electron ionization (EI) mode. Insets show an expansion of the area containing the base peak and the labeled isomer. (E) Major peaks in the spectra and their sources.

**Fig. 1.** Incorporation of $^{13}$C and $^{15}$N from asparagine into isoleucine in *Acyrthosiphon pisum*. Aphids were fed on a diet containing $[U-^{13}$C, $U-^{15}$N]asparagine, amino acids were extracted by acid hydrolysis, and isoleucine was detected by GC-MS. (A) Metabolic pathway for the biosynthesis of isoleucine from asparagine, with 2-oxobutanoic acid as an intermediate. M+1 atoms ($^{13}$C and $^{15}$N) are marked with an asterisk. Isoleucine made from $[U-^{13}$C, $U-^{15}$N]asparagine has a predicted +4 mass increase, +5 if the nitrogen is also derived from the breakdown products of labeled dietary asparagine. (B–E) Mass spectra and representative structures that were analyzed. (B) Chemical ionization (CI) mode mass spectrum of isoleucine produced from $[U-^{13}$C, $U-^{15}$N]asparagine, showing incorporation of either four $^{13}$C and one $^{15}$N atom, or one $^{15}$N atom (m/z 281 and 277, respectively). Inset shows an expansion of the area containing the molecular ion peaks of the labeled isomers. (C,D) Mass spectra of unlabeled and labeled isoleucine produced from $[U-^{13}$C, $U-^{15}$N]asparagine, respectively, showing incorporation of one $^{15}$N atom in electron ionization (EI) mode. Insets show an expansion of the area containing the base peak and the labeled isomer. (E) Major peaks in the spectra and their sources.

**Fig. 2.** Incorporation of nitrogen from phloem transport amino acids into other amino acids in *A. pisum*. Aphids were fed the indicated amino acid diets, balanced to pH 7. Abundance of the EI-MS fragment [FM+1] ion ([FM=117, loss of COOTMS]) resulting from $^{15}$N or a single $^{13}$C carbon atom incorporation is shown as a percentage of the abundance of the unlabeled molecule in the same samples, i.e. a value of 100 indicates that ions for [FM+1] and [FM] are equally abundant. The total amino acid component of the samples was prepared by hydrolysis with 6 mol l$^{-1}$ HCl. Under this condition, asparagine and glutamine are hydrolyzed to the respective acids. Therefore, we cannot differentiate the aspartic acid and asparagine, or glutamic acid and glutamine, content of the samples. Methionine was detected only in some samples, hence it was not included in the analysis of the data, but it also showed an increase of 1 mass unit for the fragment ion [M-117]. Each bar represents the mean and standard error of 5–9 samples. For each amino acid, different letters indicate significant differences (P<0.05 ANOVA followed by Tukey’s HSD test).
U^{15}\text{N}\text{asparagine diet contained sevenfold more }^{15}\text{N than the }[U^{13}\text{C}, U^{15}\text{N}]\text{aspartic acid diet, and the }[U^{13}\text{C}, U^{15}\text{N}]\text{glutamic acid diet contained fourfold more }^{15}\text{N than the }[U^{13}\text{C}, U^{15}\text{N}]\text{glutamic acid diet (Table 1). This could explain the lower incorporation of labeled nitrogen in the aphids fed on }^{15}\text{N}\text{aspartic acid and }^{15}\text{N}\text{glutamic acid diets compared with those on the }[U^{13}\text{C}, U^{15}\text{N}]\text{asparagine or }[U^{13}\text{C}, U^{15}\text{N}]\text{glutamine diets (Fig. 2).}

To confirm that the mass increase [M+1-117] was due to the labeled nitrogen addition, we conducted experiments with diets that contained [U^{15}\text{N}]\text{asparagine or }[U^{15}\text{N}]\text{glutamine, together with unlabeled aspartic acid and glutamic acid, respectively. Analysis of amino acids from aphids fed on these diets showed that, in all amino acids detected, there was incorporation of labeled nitrogen that caused [M+1] increases in the nitrogen-containing fragment [M-117, M-COOTMS] (Fig. 3). However, there were differences caused [M+1] increases in the nitrogen-containing fragment [M-117] with the [U-13C, U-15N]-labeled diets (Fig. 2) than with the [U-15N]-labeled diets (Fig. 3), suggesting that, in addition to the nitrogen, a carbon from the labeled amino acids may be incorporated. Other labeling differences could be due to unexplained variation in the phenology of the aphids at the time of processing in the respective experiments.

Differential incorporation of α- and γ-nitrogen from asparagine
To determine whether the two nitrogen atoms of asparagine are incorporated in the same manner by the A. pisum–Buchnera system, we conducted artificial diet experiments with specifically labeled [α-15N]asparagine and [γ-15N]asparagine. The nitrogen incorporation from the diet containing [U-15N]\text{asparagine was higher than that with either [α-15N]asparagine or [γ-15N]asparagine, indicating that both nitrogens from [U-15N]\text{asparagine can be incorporated (Fig. 3). However, in most cases, there was significantly greater incorporation of the α-nitrogen than the γ-nitrogen of asparagine into other amino acids. Although the two nitrogen atoms of dietary asparagine are not functionally equivalent during A. pisum amino acid biosynthesis, they were incorporated similarly in the biosynthesis of the nucleic acids uracil and thymine (Fig. S3).}

Fate and conversion of the carbon from transport amino acids into other amino acids
We conducted isotopomer analysis of all of the detected amino acids, choosing those fragments of the mass spectrum that were the base peaks and retained the nitrogen atom of the amine group. For example, for aspartic acid, we chose the fragment ion m/z 232 and, if all the carbons and nitrogen atoms were retained, then we would see an isotopomer for m/z 232+4. Similarly, for glutamine (pyroglutamic acid after acid hydrolysis), we chose fragment ion m/z 156 and, if all of the labeled carbon and nitrogen atoms were retained, we would observe an isotopomer at m/z 156+5. If only nitrogen were to be labeled, then we would see m/z 232+1 and 156+1 for aspartic acid and glutamic acid, respectively (Fig. 4). If we observed the isotopomers with masses between the fully labeled and nitrogen-labeled isotopomers, it would suggest that the labeled carbon atoms are also incorporated into these amino acid isotopomers. In that case, there would be various combinations of labeled atoms being incorporated into the various locations. Examples of possible combinations of labeled nitrogen and carbon for aspartic acid and pyroglutamic acid are shown in Fig. S1.

The EI-MS isotopomer analysis of the dietary amino acids in the aphids showed that a only a small percentage of aspartic acid/asparagine has an intact, isotope-labeled carbon backbone (FM+ 4 and, if all of the labeled carbon and nitrogen atoms were retained, we would see an isotopomer for m/z 232+4. Similarly, for glutamine (pyroglutamic acid after acid hydrolysis), we chose fragment ion m/z 156 and, if all of the labeled carbon and nitrogen atoms were retained, we would observe an isotopomer at m/z 156+5. If only nitrogen were to be labeled, then we would see m/z 232+1 and 156+1 for aspartic acid and glutamic acid, respectively (Fig. 4). If we observed the isotopomers with masses between the fully labeled and nitrogen-labeled isotopomers, it would suggest that the labeled carbon atoms are also incorporated into these amino acid isotopomers. In that case, there would be various combinations of labeled atoms being incorporated into the various locations. Examples of possible combinations of labeled nitrogen and carbon for aspartic acid and pyroglutamic acid are shown in Fig. S1.

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monoisotopomer) for diets containing [U-13C, U-15N]glutamine and [U-13C, U-15N]asparagine, respectively (Fig. 4A). In contrast, somewhat more glutamic acid and glutamine were observed as direct diet-derived amino acids (FM+5 was about 12% of the total isotopomers or 28% of the monoisotopomer) (Fig. 4B). There are some limitations to this assay: (i) we cannot determine whether these isotopomers came from unreacted glutamine and asparagine or whether they were resynthesized after degradation to keto-acids; (ii) as decarboxylated amino acid fragments are being measured in this assay, it is not possible to determine whether the carboxyl carbon atom is 13C-labeled in the measured amino acids; (iii) as total amino acids were prepared by acid hydrolysis, asparagine and glutamine were converted to their respective acids, and we cannot determine whether glutamic acid and aspartic acid are synthesized directly from asparagine or glutamine, respectively, by the aphids; and (iv) at any given time some isotope-labeled dietary amino acids are still in the gut and have not yet been metabolized by the aphids.

To determine whether there is de novo synthesis of otherwise abundant dietary amino acids in the aphid–Buchnera system, we measured the abundance of other isotopomers. In addition to the higher amounts of FM+1 (15N incorporation; Fig. 2) and FM+4 (all 13C and 15N retained; Fig. 4A), we observed significant amounts of the FM+2 and FM+3 isotopomers for aspartic acid/asparagine in aphids that were fed on the [U-13C, U-15N]asparagine and [U-13C, U-15N]glutamine diets (Fig. 4A). Similarly, relative to control samples, we measured increased amounts of FM+2, FM+3 and FM+4 for the [U-13C, U-15N]glutamic acid and [U-13C, U-15N]glutamine diets (Fig. 4B). The presence of variable numbers of 13C-labeled carbon atoms suggests breakdown of the dietary amino acids and de novo synthesis that reincorporates some
of the isotope-labeled atoms. In fact, the relatively high abundance of these isotopomers shows that most of the dietary amino acids are catabolized and re-synthesized.

Significant incorporation of the carbon backbone from the dietary amino acids was detected in isoleucine and threonine (Fig. 4C,D). In the respective experiments, the abundance of FM+4 labeled isoleucine, expressed as a percentage of native unlabeled isoleucine, was 15% (7.2% of total isotopomers) for [U-13C, U-15N]asparagine and 20% (7.2% of total isotopomers) for [U-13C, U-15N]glutamine. In the case of threonine, the results were similar, with incorporation efficiencies of approximately 11% (5% of total isotopomers) from [U-13C, U-15N]asparagine and 33% (12% of total isotopomers) for [U-13C, U-15N]glutamine of native unlabeled threonine. In contrast, aphids fed on [U-13C, 15N]aspartic acid or [U-13C, 15N]glutamic acid diet did not show any significant incorporation of the amino acid carbon skeleton relative to control aphids reared on unlabeled diet. The isotopomer analysis also showed that there was addition of other numbers of labeled carbon atoms (FM+2 or FM+3) into isoleucine and threonine (Fig. 4C,D), indicating that the backbone carbon chain of these amino acids can be synthesized with carbon atoms that were derived from degraded dietary asparagine or glutamine.

We also observed incorporation of isotope-labeled carbon atoms into leucine, lysine, phenylalanine, valine, alanine, tyrosine and serine (Fig. 4E–K). Almost all of these amino acids showed some incorporation of two carbon atoms or one carbon and one nitrogen, but the amounts varied for different dietary inputs. In most cases, the highest incorporation was from [U-13C, U-15N]asparagine and [U-13C, U-15N]glutamine. Phenylalanine and tyrosine incorporated higher amounts of M+2 from the [U-13C, U-15N]glutamine diet than from the [U-13C, U-15N]asparagine diet. The possibility of extremely small amounts of intact carbon chain incorporated into other amino acids cannot be ruled out as we did not carry out isotopomer analysis for all the amino acids if the percentage was very small (less than 2%). Even if this did happen, the contribution to total amino acid content is likely to be negligible. In the case of glycine (Fig. 4L), there was no evidence of 13C incorporation from any of the [U-13C, U-15N]-labeled dietary amino acids.

Compared with the other measured amino acids (Fig. 2), proline was unusual in that the FM+1 isotopomer from aphids that had been fed with the diets containing [U-13C, 15N]aspartic acid, [U-13C, 15N]glutamic acid and [U-13C, U-15N]glutamine was severalfold higher than FM (up to 800% of FM; Fig. 5A). However, the [U-13C, U-15N]asparagine diet showed FM+1 as only about 97% of FM. This suggested that either much of the FM+1 isotopomer came from (i) the higher conversion of 15N-labeled proline from the respective keto-acids or (ii) incorporation of a single labeled 13C from the universally labeled respective dietary amino acids. To determine whether the contribution of +1 mass comes from labeled nitrogen and/or carbon, we analyzed the contribution of +1 mass of aphids that were fed with [15N]-labeled diets (Fig. 5B). In each case, the FM+1 isotopomer was much less abundant than with the [U-13C, 15N]aspartic acid, [U-13C, 15N]glutamic acid and [U-13C, U-15N]glutamine diets, indicating that the elevated FM+1 in aphids that were fed on universally labeled diet comes from the incorporation of a labeled carbon (13C) in addition to labeled nitrogen (15N). The relatively low proline FM+1 isotopomer from [U-13C, U-15N]asparagine (Fig. 5A) abundance suggests that only 15N is incorporated and that the four dietary amino acids are utilized differently by the aphid–Buchnera system in proline biosynthesis.

**DISCUSSION**

Metabolic models based on the presence and absence of amino acid biosynthesis genes in *A. pisum* and *Buchnera*, as well as amino acid uptake and labeling experiments, show that precursors for the biosynthesis of essential amino acids are transported from the aphid host to the bacterial endosymbionts (Brinza et al., 2010; Hansen and Moran, 2011; Poliakov et al., 2011; Russell et al., 2014; Sasaki and Ishikawa, 1995; Shigenobu et al., 2000; Wilson et al., 2010). Four amino acids, aspartic acid, asparagine, glutamic acid and glutamine, not only provide the majority of the nitrogen flow into the *Buchnera* endosymbionts for the synthesis of essential amino acids but also constitute the majority of the phloem amino acids in most plant species, including in *V. faba*, a natural host for *A. pisum* (Gündüz and Douglas, 2009). These observations, together with aphid feeding experiments using radioactive metabolites (Sasaki and Ishikawa, 1995), suggested that dietary amino acids can be moved intact from the insect gut to the bacterial endosymbionts to serve as precursors for the synthesis of other amino acids.

For most of the amino acids detected in our experiments, the predominant isotopomers were M+1 (Fig. 2), likely indicating incorporation of 15N into amino acids through transamination of...
Proline is unusual among the detected aphid amino acids in that it is one of the sugar is excreted in the honeydew. Thus, there will be an enrichment of amino acid-derived carbon atoms in the aphid bodies relative to the diet from which they are feeding.

The variability of the aphid dietary amino acid content may explain the propensity for the de novo synthesis of even amino acids that are overabundant in the diet. The composition of phloem amino acids in plants depends on the type of nitrogen available to the roots, oxygen levels, plant symbionts, the time of day, and other environmental conditions (Coruzzi and Last, 2000; Liu et al., 2013; Oliveira et al., 2013). Moreover, several studies have demonstrated that aphid-infested plants have altered amino acid composition (Leroy et al., 2011; Liadouze et al., 1995; Sandström and Moran, 2001; Sasaki et al., 1990). Thus, the A. pisum–Buchnera system may have evolved to efficiently assimilate multiple available phloem nitrogen resources to produce other amino acids. Maximum flexibility in the utilization of dietary nitrogen might be provided through the catabolism and re-assembly of dietary amino acids, as needed, rather than relying on the variable abundance of particular dietary amino acids for transport of metabolic precursors to the Buchnera endosymbionts.

Proline is unusual among the detected aphid amino acids in that there is an eightfold higher ratio of a single $^{13}$C incorporated from dietary sources. This could be an indication that proline has a higher turnover than other aphid amino acids, thereby allowing a higher labeling specificity. In addition to being an essential component of proteins, proline has an osmoprotective function in...
many organisms, ranging from yeast to arthropods (Kostal et al., 2011; Takagi, 2008; Verslues and Sharma, 2010). As osmoregulation is a unique challenge faced by aphids feeding from phloem sap containing roughly 1 mol L⁻¹ sucrose (Douglas, 2006), there may be aspects of proline metabolism and turnover that make synthesis of this amino acid different from that of other amino acids that were detected in our GC-MS assays.

Together, our results provide new insight into amino acid biosynthesis by A. pisum and Buchnera symbiosis. Notable findings include: (i) the catabolism and re-synthesis of dietary amino acids; (ii) the high level of de novo synthesis of non-essential amino acids, even when they are presumably overabundant in the diet; and (iii) the variable incorporation of different nitrogen sources into essential amino acids. The supply of precursors for synthesis of essential amino acids by Buchnera is clearly more complex than simply the transport of dietary amino acids from the gut to the endosymbionts. As all animals interact with microbes during their growth and development, the provisioning of amino acids by commensal microbes is a more general phenomenon. Thus, research on the shared amino acid biosynthesis of aphids and their bacterial endosymbionts may have implications beyond this specific biological system.

Acknowledgements

We thank A. E. Douglas for providing the CWR09/18 aphid clone and the modified aphid diet composition.

Competing interests

The authors declare no competing or financial interests.

Author contributions

M.H. designed the experiments, performed the research, analyzed the data and wrote the manuscript. G.J. designed the experiments, analyzed the data and wrote the manuscript.

Funding

This research was funded by the US National Science Foundation [grant number IOS-0919765] and US Department of Agriculture [grant number 2012-67013-19350].

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.129189/-/DC1

References


Figure S1. A. Examples of isotopomers of aspartic and pyroglutamic acid fragment ions monitored. Theoretical values of m/z for unlabeled fragment ion mass are: FM [M-116]: 232.12(100.0%), 233.12(10.2%), 234.12 (9.7%). The numbers below indicate possible combinations of isotopomer positions of three labeled carbon atoms and a labeled nitrogen atom for each isotopomer.

B. Isotopomers of pyroglutamic acid fragment, ions monitored. Theoretical values of m/z for unlabeled fragment ion are: FM [M-116]: 156.08 (100.0%), 157.08 (7.6%), 158.08(5.1%). The numbers below indicate possible combinations of isotopomer positions of three labeled carbon atoms and a labeled nitrogen atom for each isotopomer of pyroglutamic acid.
**Figure S2. Aphid growth on different diets.** Aphids were grown on *V. faba* until they were two days old (control samples), at which point they were moved to the indicated artificial diets for five additional days. Mean +/- s.e. of N = 4 to 17. *P < 0.05, two-tailed t-test relative to control sample of 2 day old plant reared aphids.
Figure S3. Incorporation of $^{15}$N and $^{13}$C into uracil and thymine from the indicated diets. Aphids fed on all diets show incorporation of at least one atom (M+1), most likely a nitrogen. In many cases, for universally labeled amino acids, M+2 was also significantly higher in uracil and thymine. This indicates that there is either addition of two nitrogen atoms or a combination of nitrogen and carbon atoms. Each bar represents the mean and standard error of 5 to 9 samples. The fragment ions of the same isotopomers in different treatments are denoted by the same color. Different letters indicate significant differences, $P < 0.05$ ANOVA followed by Tukey’s HSD test.
Figure S4: Annotated and predicted pathways to amination and deamination in the *Acyrthosiphon pisum – Buchnera* system. (A) Pathways in the KEGG database. (B) Transamination predicted based on the presence of aphid genes. Pink arrows - annotated pathways in *Buchnera*; Blue arrows - annotated pathways in *A. pisum*; Red arrows - predicated pathways based on gene annotation.
**Supplementary Table S1.** Artificial diet for *Acyrthosiphon pisum* feeding experiments

Five (A-E) solutions were prepared and mixed as indicated below and diluted with water to bring to desired dilution. All weights are in mg. For labeled diet the amounts were based on respective isotope composition.

### Solution A

**Amino acids dissolved in 50 ml of double distilled water**

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Complete diet</th>
<th>Aspartic acid + Asparagine diet</th>
<th>Glutamic acid + Glutamine diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>50.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>300.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>213.9</td>
<td>836.5</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>189.7</td>
<td>220.9</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>42.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>241.1</td>
<td>723.1</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>123.6</td>
<td>370.7</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>182.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>114.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>114.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>158.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>42.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl alanine</td>
<td>47.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>65.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>59.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>103.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>58.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>10.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>101.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Solution B

**Minerals**

Dissolved in 10 ml of double distilled water

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl3. 6H2O</td>
<td>11</td>
</tr>
<tr>
<td>CuCl2.4H2O</td>
<td>2</td>
</tr>
<tr>
<td>MnCl2.6H2O</td>
<td>4</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>17</td>
</tr>
</tbody>
</table>
Solution C

*Vitamins* dissolved in 5 ml of double distilled water

- Biotin: 0.1
- Pantothenate: 5
- folic acid: 2
- nicotinic acid: 10
- pyridoxine: 2.5
- thiamine: 2.5
- choline: 50
- myo-inositol: 50

Solution D

*Sugars and acids* dissolved in 3 ml of double distilled water

- Ascorbic acid: 10
- Citric acid: 1
- MgSO4.7H2O: 20
- Sucrose: 1700

Solution E

*Phosphates* dissolved in 1 ml of double distilled water

- K2HPO4: 115

To make 10 ml of diet mix the solutions A-E in following ratio and make sure your pH is 7

- Solution A: 5.0 ml
- Solution B: 0.1 ml
- Solution C: 0.5 ml
- Solution D: 3.0 ml
- Solution E: 1.0 ml
- Water: 0.4 ml