The Journal of Experimental Biology 204, 349–358 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3120

QUANTIFYING NUTRIENT PRODUCTION BY THE MICROBIAL SYMBIONTS IN AN APHID

A. E. DOUGLAS, L. B. MINTO AND T. L. WILKINSON*

Department of Biology, University of York, York YO10 5YW, UK

*Author for correspondence and present address: School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK (e-mail: T.L.Wilkinson@bham.ac.uk)

Accepted 3 November 2000; published on WWW 3 January 2001

Summary

The symbiotic bacteria *Buchnera* sp. provide aphids with essential amino acids, nutrients in short supply in the aphid diet of plant phloem sap. The contribution of *Buchnera*-derived amino acids to net protein growth of the aphid *Aphis fabae* was quantified from the protein growth of aphids reared on chemically defined diets lacking individual amino acids. The amino acid production rates varied among the nine essential amino acids over the range 8–156 pmol μg^{-1} protein day^{-1} (for tryptophan and leucine, respectively), equivalent to 0.02–0.33 fmol *Buchnera*$^{-1}$ day^{-1}. In a complementary metabolic analysis, the aphids incorporated radioactivity from dietary [^{14}C]glutamic acid into the essential amino acids isoleucine, lysine and threonine. Incorporation into isoleucine was significantly elevated by the omission of dietary isoleucine, indicating that dietary supply may affect the biosynthetic rates of certain amino acids by *Buchnera*. Aphids experimentally deprived of *Buchnera* did not synthesize essential amino acids from dietary glutamic acid. The mortality of aposymbionts was high over 7 days on the phenylalanine-free diet, and their assimilation of dietary leucine was depressed on the complete diet, suggesting that both the absence of bacteria-derived amino acids and the low rates of assimilation of certain dietary amino acids may contribute to the poor growth of these insects.

Key words: *Buchnera*, *Aphis fabae*, aphid, symbiosis, amino acid, nutrition, Homoptera, protein growth.

Introduction

Feeding on plant sap has evolved rarely among animals (Raven, 1983). A contributory reason is that the nitrogenous compounds in plant sap are dominated by one or a few amino acids, with very low concentrations of the nine amino acids that animals cannot synthesize *de novo* (Douglas, 1993; Sandström and Pettersson, 1994; Sandström and Moran, 1999). [These 'core' essential amino acids are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Morris, 1991).] Exceptionally, most insects in the order Homoptera (aphids, whitefly, planthoppers, leafhoppers, cicadas, etc.) feed exclusively on plant phloem or xylem sap. Among these insects, symbiosis with micro-organisms is universal (Buchner, 1966; Douglas, 1989). The micro-organisms in aphids, bacteria of the genus *Buchnera* in the division γ-Proteobacteria (Munson et al., 1991), have been shown to provide the insect with the essential amino acids in short supply in phloem sap, such that aphids are partially or completely independent of a dietary supply of these nutrients (Douglas, 1998). The *Buchnera* are located in specialised insect cells, known as mycetocytes or bacteriocytes, in the aphid body cavity and they are transmitted to aphid offspring *via* the ovary (Buchner, 1966).

The evidence that *Buchnera* contributes amino acids to aphids comes principally from physiological experiments comparing the amino acid biosynthetic capabilities and nutritional requirements of untreated aphids containing *Buchnera* (symbiotic aphids) and aphids experimentally deprived of their bacteria, usually by antibiotic treatment (aposymbiotic aphids) (Douglas, 1998; Wilkinson, 1998). For example, symbiotic, but not aposymbiotic, *Myzus persicae* can incorporate ^{35}S from dietary inorganic sulphate into methionine (Douglas, 1988); symbiotic, but not aposymbiotic, *Acyrthosiphon pisum* synthesize the essentials isoleucine, lysine and threonine from dietary [^{14}C]glutamic acid and all essential amino acids from dietary [^{14}C]sucrose (Febvay et al., 1995; Febvay et al., 1999); and the concentrations of several essential amino acids in the free amino acid fraction of *Ac. pisum* are depressed by aposymbiosis (Prosser and Douglas, 1991; Liadouze et al., 1995). Spectacular confirmation of these physiological data comes from molecular analysis of the *Buchnera* genome. In particular, the recently completed full genome sequence of *Buchnera* in *Ac. pisum* (Shigenobu et al., 2000) has demonstrated conclusively that *Buchnera* has a much reduced genome and yet has retained the genes for the biosynthesis of most essential amino acids (also see Moran and Baumann, 2000; Charles and Ishikawa, 1999; Baumann et al., 1995).

Although the experimental evidence for bacterial provision of essential amino acids is persuasive, the data are largely qualitative. The rates of essential amino acid synthesis by *Buchnera* are widely assumed to be high, but there is only one published quantitative estimate of nutrient transfer in this symbiosis: the minimal estimate of 0.02 fmol of tryptophan per *Buchnera* cell per day, based on the rate of tryptophan excretion by symbiotic *Ac. pisum* (value from Douglas and Prosser, 1992; recalculated to correct the twofold underestimation of *Buchnera* density in larval *Ac. pisum*; see Humphreys and Douglas, 1997).

The broad purpose of this study was to quantify the rates of essential amino acid production in the aphid–*Buchnera* symbiosis. In this context, the 'essential' amino acids are defined as the nine amino acids (see above) that an aphid has no capacity to synthesize *de novo* and are therefore required absolutely from the diet or from symbiotic bacteria. The experiments were conducted on aphids reared on chemically defined diets, so that the dietary concentrations of amino acids were known and could be manipulated precisely. Most recent research on essential amino acid synthesis in the aphid symbiosis has been conducted with *Ac. pisum* (for a review, see Wilkinson, 1998), but the experimental design of dietary studies on this species is constrained by the rejection of diets by all plant-reared *Ac. pisum* except very young larvae. To circumvent these limitations, this study was conducted on the black bean aphid, *Aphis fabae* Scop., all ages of which accept diets readily (A. E. Douglas, unpublished results). Microscopical and molecular analyses have confirmed that *Buchnera* is the dominant micro-organism in *Aph. fabae* (A. E. Douglas, unpublished data; B. Raymond, J. B. Searle and A. E. Douglas, in preparation), and larvae of *Aph. fabae* reared on diets individually lacking every essential amino acid except methionine or histidine developed normally to adulthood (Leckstein and Llewellyn, 1973), suggesting that the symbiosis may contribute to the aphid requirements for most essential amino acids. However, essential amino acid biosynthesis by the *Aph. fabae* symbiosis has not been demonstrated directly.

The basis of our approach to quantify amino acid production was that the amino acid composition of aphid protein does not vary with diet composition or aposymbiosis (see Materials and methods) such that the net rate of increase of each essential amino acid in the protein fraction could be determined from the protein growth rate of the aphid. On a chemically defined diet lacking a single essential amino acid, the sources of that amino acid for net protein growth are *de novo* synthesis by *Buchnera* in symbiotic aphids and preformed reserves in the free amino acid fraction of both symbiotic and aposymbiotic aphids. The incorporation rates of *Buchnera*-derived essential amino acids into protein can, therefore, be calculated from the difference between protein growth rates of symbiotic and aposymbiotic aphids. This dietary analysis was complemented by metabolic studies designed to explore further the basis of the low growth rates of aposymbiotic aphids and to assess the impact of dietary supply of essential amino acids on *Buchnera*-mediated biosynthetic rates.

Materials and methods

The chemically defined diets

The composition of the complete diet was formulation A (see Prosser and Douglas, 1992) with 0.5 mol l^{-1} sucrose and 0.15 mol l^{-1} amino acids (16.5 mmol l^{-1} glutamine, 14.3 mmol l^{-1} asparagine, 14.3 mmol l^{-1} aspartic acid, 14.3 mmol l^{-1} arginine, 8.7 mmol l^{-1} histidine, 8.7 mmol l^{-1} isoleucine, 8.7 mmol l^{-1} leucine, 8.7 mmol l^{-1} lysine, 8.7 mmol l^{-1} threonine, 8.7 mmol l^{-1} valine, 8.4 mmol l^{-1} glutamic acid, 5.7 mmol l^{-1} alanine, 5.7 mmol l^{-1} proline, 5.7 mmol l^{-1} serine, 2.9 mmol l^{-1} methionine, 2.9 mmol l^{-1} phenylalanine, 2.9 mmol l^{-1} tryptophan, 2.7 mmol l^{-1} cysteine, 1.2 mmol l^{-1} glycine and 0.6 mmol l^{-1} tyrosine). Two types of dietary modification were used: addition of the antibiotic rifampicin at 50 μg ml^{-1} (Rahbé et al., 1994) to the complete diet; and individual omission of each of the 20 amino acids, such that the total amino acid concentrations of the diets were 133.5–149.4 mmol l^{-1}. Diets were also supplemented with radioactive compounds: L-[U-^{14}C]glutamic acid (ICN Radiochemicals Ltd, 1 mCi ml^{-1}) at 3.7 MBq ml^{-1} diet; L-[U-^{14}C]leucine (Sigma Chemical Co., 3.7 MBq ml^{-1}) at 30.7×10^{-5} MBq ml^{-1} diet; and [^{3}H]inulin (Sigma, 37 MBq ml^{-1}) at 27.8×10^{-5} MBq ml^{-1} diet. The chemical purity of radiochemicals was confirmed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Parafilm sachets of filter-sterilized diet were prepared (as described by Douglas, 1988) and applied across one end of diet cages, Perspex cylinders of internal diameter 3.5 cm.

The aphids and experimental designs

A parthenogenetic culture of *Aphis fabae* Scop. clone HR91/3 was raised from a single female collected from a *Vicia faba* crop at Abingdon, Oxon, UK, in summer 1991 and maintained at low density on pre-flowering *V. faba* cv. The Sutton. The conditions for aphid culture and all experiments were 20 °C with a 18 h:6 h light:dark photoperiod.

The experimental designs were informed by preliminary data on the performance of clone HR91/3 on chemically defined diets (A. E. Douglas, unpublished results): the mean relative growth rates of larvae reared in groups of 5–10 on the complete diet was 0.3 mg mg^{-1} day^{-1}, a value comparable with growth rates on plants (Adams and Douglas, 1997), but was depressed by 15 % for aphids reared singly; aphid survival and weight gain did not vary significantly on the complete diet with amino acid concentrations in the range 0.10–0.15 mol l^{-1}; the population of *Buchnera* was disrupted by rifampicin treatment for 2 days, as revealed by microscopical analysis of serially sectioned aphids; and alate production, promoted by certain amino acid deletions (see also Leckstein and Llewellyn, 1973), was completely abolished by maintaining insects on the complete diet for the first 2 days of larval development and subsequently raising them in isolation.

All experiments were initiated by transferring adult apterae from plants to the complete diet either containing or lacking rifampicin. Larvae deposited over 1 day were retained on the diets for a second day. These aphids are termed 2 days old,

and those on diets with and without rifampicin are called 'aposymbiotic' and 'symbiotic' aphids, respectively. Unless stated otherwise, groups of five of these 2-day-old aphids were transferred to rifampicin-free diets (either complete or with an individual amino acid omission) for 7 days (i.e. to 9 days old). For experiments on the metabolism of [^{14}C]glutamate, five replicate groups of aphids were reared on the radiolabelled diets for 7 days, with two control groups on radioactive-free diets for each treatment.

The radiolabelled inulin technique (Wright et al., 1985; Wilkinson and Ishikawa, 1999) was used to quantify aphid feeding rates and assimilation of dietary leucine. Twelve replicate symbiotic and aposymbiotic aphids reared to 7 days on the complete diet were transferred individually to a sachet of complete diet supplemented with [^{14}C]leucine and [^{3}H]inulin on a Perspex ring (diameter 3.5 cm, height 0.5 cm) above a 3.5 cm diameter GF/C filter (Whatman), with aphids on duplicate non-radioactive sachets as controls. Inulin is not degraded or assimiliated by *Aph. fabae* (L. B. Minto and A. E. Douglas, unpublished results), and the ^{3}H and ^{14}C contents of the honeydew deposited on the filter were quantified by scintillation counting (see below), from which the total volume of diet ingested and the amount of dietary leucine egested per aphid, respectively, were obtained.

To quantify the density of *Buchnera* cells in aphids, individual aphids were homogenized in a known volume of ice-cold 50 mmol l^{-1} Tris-HCl, pH 7.5, with 0.25 mol l^{-1} sucrose and 3.9×10^{7} polystyrene 'ultraspheres' ml^{-1} (sphere diameter 2.1 μm) (final density). Eight replicate samples of each homogenate were examined by phase-contrast microscopy at ×1000 magnification, and the numbers of *Buchnera* cells and ultraspheres in a single field of view per sample were scored. The volume of homogenate per field of view was calculated from the number of ultraspheres scored, and this was used to transform the number of *Buchnera* cells scored to *Buchnera* density following determination of the protein content of each homogenate (see below).

Chemical analyses

The free amino acids of aphids were extracted by homogenizing individual 9-day-old insects in 0.1 ml of ice-cold 80 % methanol in a hand-held glass tissue grinder. The homogenate was centrifuged at 20 000 ***g*** for 15 min and the supernatant retained. Protein hydrolysates of the aphids were obtained by acid hydrolysis. Each replicate group of aphids was homogenised in 0.2 ml of ice-cold 0.1 mol l^{-1} phosphate buffer, pH 7.0, brought to 0.45 ml with buffer, extracted with 10 % (v/v final concentration) ice-cold trichloroacetic acid (TCA) for 30 min and centrifuged at 20 000 ***g*** for 15 min at 4 °C. The pellet was washed twice with 10 % TCA and then with ether (to remove TCA), dried and hydrolysed in 6 mol l^{-1} HCl at 110 °C for 24 h in a sealed ampoule. The hydrolysate was neutralised with NaOH, dried in a Speed-Vac and dissolved in 80 % methanol.

The amino acids in methanol extracts and protein hydrolysates of the aphids were quantified by reverse-phase HPLC after derivatization with *o*-phthaldialdehyde (following the procedure of Jones et al., 1981) using a Beckman System Gold delivery system with C_{18}-ultrasphere column and Shimadzu RF-551 fluorescence detector. This procedure can detect all protein amino acids except cysteine and proline (in some experiments, histidine and serine could not be separated). The reference amino acids were AA-S-18 (Sigma), supplemented with asparagine, glutamine and tryptophan. All detectable protein amino acids were quantitatively recovered from the protein hydrolysates except tryptophan, which was degraded, and asparagine and glutamine, which were hydrolysed to aspartic acid and glutamic acid, respectively. Preliminary experiments confirmed that the amino acid composition of protein hydrolysates did not vary significantly with either aposymbiosis or amino acid omissions from the diet (tested for diets lacking isoleucine, leucine and threonine), and the mean amino acid composition of *Aph. fabae* protein is shown in Table 1.

The protein content of aphid homogenates was quantified by the microassay method of BioRad, following the manufacturer's instructions, with bovine serum albumin as standard. The mass of aphids was determined to the nearest microgram on a Mettler MT5 microbalance. In test samples of diet-reared *Aph. fabae*, the protein content per unit fresh mass was 60 μg mg^{-1} for 2-day-old aphids and 53 and 38 μg mg^{-1} for 9-day-old symbiotic and aposymbiotic aphids, respectively. Aphids were weighed individually, and their protein contents obtained by calculation where the experimental design precluded killing the aphids (e.g. the initial protein content of 2-day-old aphids used for assays of protein growth rates).

Radiochemical analyses

The incorporation of radioactivity into amino acids of the TCA-soluble fraction and protein hydrolysates of aphids was assessed by HPLC and TLC. The eluate from the HPLC delivery system was collected with a fraction collector (30 s per vial) and quantified by scintillation counting (see below). Colocalization of the radioactivity and amino acid peaks was confirmed by spiking samples with ^{14}C-labelled amino acids ([^{14}C]glycine and [^{14}C]leucine were particularly helpful for discriminating the ^{14}C content of the amino acid pairs glycine/threonine and isoleucine/leucine with close but distinct elution times). For TLC, samples were applied to silica plates (HPTLC, Merck Co.), run in methanol:chloroform:17.5 % ammonia (2:2:1) in the first dimension and phenol:water (3:1) in the second dimension, and then exposed to X-ray film (Sigma) for 4–7 days. Radiolabelled spots were colocalized with amino acids visualised by ninhydrin, as confirmed by comparison with standards.

To quantify the radioactivity in aphid honeydew and crude homogenates, TCA extracts and protein hydrolysates of aphids, 4 ml of scintillation fluid (Ultima Gold XR, Packman) was added to samples, and ^{14}C content was determined in a scintillation counter (Tri-carb, Packman) with preset ^{14}C or ^{3}H/^{14}C dual windows, as appropriate, and quench curve. The

Table 1. *Amino acid content of protein in* Aphis fabae

Amino acid	Percentage composition (g)	Percentage composition (mol)
Alanine	3.8	5.6
Arginine	3.5	2.6
Asx[1]	13.1	12.9
Cysteine[2]	1.6	1.7
Glx[1]	15.0	13.3
Glycine	2.4	4.2
Histidine	1.0	0.9
Isoleucine	6.6	6.5
Leucine	10.6	10.5
Lysine	9.7	8.6
Methionine	2.8	2.4
Phenylalanine	5.8	4.6
Proline[2]	5.6	6.3
Serine	3.7	4.6
Threonine	5.6	6.1
Tryptophan[2]	0.8	0.5
Tyrosine	1.5	1.1
Valine	6.9	7.6

[1]Combined concentrations of asparagine plus aspartic acid (Asx) and glutamine plus glutamic acid (Glx).

[2]These amino acids could not be quantified empirically; published values for insects (Vegotsky and Fox, 1962) are used, and the percentage contribution of the other amino acids was corrected for their inclusion.

counts obtained for parallel samples from the control non-radioactive aphids were subtracted from all experimental data.

Calculations and statistical analyses

The protein growth rate of each aphid reared individually from day 2 to day 9 was calculated as [ln(final protein content/initial protein content)]/7. The contribution of the *Buchnera* population to the increase in each essential amino acid was calculated as follows. Taking leucine as an example, the rate of increase in protein-leucine (i.e. rate of net leucine synthesis and incorporation into protein, μg leucine μg^{-1} protein day^{-1}) was obtained as the product of the protein growth rate (μg protein μg^{-1} protein day^{-1}) on the leucine-free diet and the leucine content of aphid protein (μg leucine μg^{-1} protein), and this value was then transformed to moles of protein-leucine synthesized (mol μg^{-1} protein day^{-1}). The contribution of *Buchnera*-derived leucine to the net increase in protein-leucine was derived by subtracting the mean rate of increase in protein-leucine in aposymbiotic aphids from that in symbiotic aphids. This analysis was repeated for each of the other eight essential amino acids.

Parametric statistical tests with critical probability P=0.05 were applied after confirmation that the data sets were normally distributed with homogeneous variances, as tested by Kolmogorov–Smirnoff one-sample test and Bartlett's test, respectively. This required logarithmic or arcsine-square root transformation of the data where indicated. The impact of aposymbiosis on the amino acid content, feeding rate and incorporation of radioactivity by aphids was tested by *t*-test. Analysis of variance (ANOVA) was applied to establish the effects of diet composition on the protein growth rates of the aphids, amino acid content and incorporation of ^{14}C from dietary glutamate into amino acids. The Bonferroni correction for multiple tests was included where appropriate, and individual differences between means were investigated by Tukey's honestly significant different method.

Results

The contribution of Buchnera*-derived amino acids to net protein growth of the aphids*

The first experiment explored the performance of larvae of *Aphis fabae* on the complete diet (containing all protein amino acids at a total concentration of 0.15 mol l^{-1}). All the symbiotic aphids and all but one aposymbiotic aphid developed to the final (fourth) larval stadium by day 9 of the experiment, as determined from exuvia number, but mortality in this stadium differed between the two groups of aphids. Pooling the data from five replicate cages of five aphids, 18/25 (72 %) symbiotic aphids and 2/25 (8 %) aposymbiotic aphids survived to adulthood (χ^2_1=12.8, P<0.001).

The analysis of aphid performance on diets from which each amino acid was individually omitted used survival and growth to day 9 of larval development as the indices of performance to ensure low mortality of aposymbiotic aphids on the complete diet. For the symbiotic aphids, 17–20 of the 20 individually caged replicate aphids survived on all diets and, among the aposymbiotic aphids, 15–20 aphids survived on all diets except the tyrosine-free and phenylalanine-free diets, on which 8/20 (40 %) and 17/20 (85 %), respectively, died. The protein growth rates of the surviving aphids are shown in Fig. 1. On every diet, the mean rate of protein growth of symbiotic aphids was greater than that of aposymbionts, varying from a twofold difference on the cysteine-free diet to a more than sevenfold difference on the leucine-free diet. The data sets for the symbiotic and aposymbiotic aphids were analysed separately because no standard transformation of the full data set generated the homogeneity of variances required for parametric statistics. Protein growth varied significantly with diet for both the symbiotic aphids ($F_{20,382}$=4.40, P<0.001) and the aposymbiotic aphids ($F_{19,330}$=4.44, P<0.001; data for the phenylalanine-free diet were excluded because of the small sample size). However, the analysis of individual differences between means by Tukey's test revealed that the pattern of aphid performance on the various diets differed between the symbiotic and aposymbiotic aphids. The protein growth rates of symbiotic aphids were significantly lower on the diets lacking the essential amino acids methionine or histidine than on the complete diet, but the performance of aposymbiotic aphids on the complete diet did not differ significantly from that on any other diet.

The protein growth rates of the aphids on diets lacking each

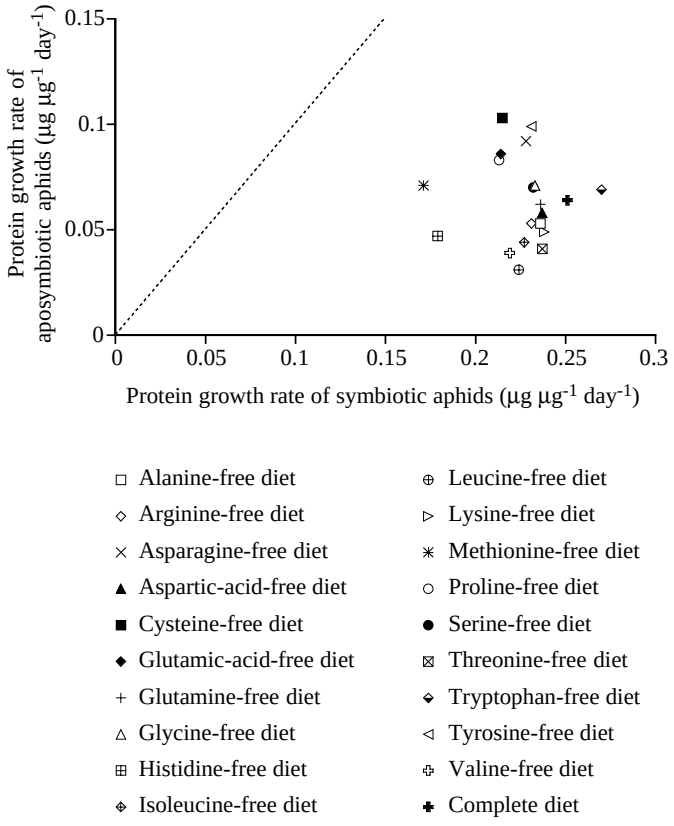

Fig. 1. Mean rate of protein growth of symbiotic and aposymbiotic *Aphis fabae* reared on diets with individual amino acid deletion from day 2 to day 9. The dotted line is the line of equivalence between symbiotic and aposymbiotic aphids. Error bars are omitted for clarity.

essential amino acid were used to estimate the amount of that amino acid synthesized by the *Buchnera* cells over the 7-day experiment (see Table 2 and calculations section of Materials and methods). The mean contribution of the *Buchnera*-derived amino acids to protein growth of symbiotic aphids varied 20-fold among the amino acids, from tryptophan and histidine at 8–9 pmol μg^{-1} protein day^{-1} to leucine at 156 pmol μg^{-1} protein day^{-1}. The density of *Buchnera* in the aphids was $4.8\times10^5\pm0.23\times10^5$ cells μg^{-1} protein (mean ± S.E.M., N=10). From these data, the amount of each amino acid synthesized by the bacteria was calculated as between 0.02 fmol (tryptophan and histidine) and 0.33 fmol (leucine) per *Buchnera* cell per day.

Amino acids in aposymbiotic aphids

The finding described above that aposymbiotic aphids on the complete diet do not perform better than on any diet lacking an individual amino acid apart from phenylalanine suggests that the dietary supply of amino acids may not replace *Buchnera*-derived amino acids. As an approach to investigate the basis of this result, the free amino acid contents of 9-day-old symbiotic and aposymbiotic *Aph. fabae* on the complete diet were quantified.

The free amino acid content of aposymbionts was elevated more than threefold, at 1.98±0.22 nmol mg^{-1} protein, relative to symbiotic aphids, at 0.59±0.049 nmol mg^{-1} protein, and the difference was statistically significant (nine replicates, t_{16}=8.93, P<0.001, on log-transformed data). The composition of the free amino acid fraction is shown in Fig. 2. Consistent with published data on aposymbiotic aphids (e.g. Prosser and Douglas, 1991; Liadouze et al., 1995; Adams et al., 1996), the asparagine and glutamine contents were particularly elevated and essential amino acid content depressed in aposymbiotic *Aph. fabae*. The essential amino acids (excluding histidine, which could not be assayed separately from serine) comprised 27±0.6 % of the total amino acid content in symbiotic and 18±0.7 % in aposymbiotic aphids (t_{16}=9.31, P<0.001 on arcsine-square root transformed data). The mean absolute

Table 2. *Net synthesis of essential amino acids by* Buchnera *in* Aphis fabae *clone HR91/3 reared on diets lacking individual essential amino acids*

	Rate of protein-amino acid synthesis (pmol μg^{-1} total protein day^{-1})		Rate of protein-amino acid synthesized by *Buchnera*	
Amino acid	Symbiotic aphids	Aposymbiotic aphids	(pmol μg^{-1} total protein day^{-1})[1]	(fmol *Buchnera* $cell^{-1}$ day^{-1})
Histidine	12.4±0.67 (20)	3.3±0.53 (19)	9.1	0.02
Isoleucine	114.7±4.34 (19)	21.9±4.36 (18)	92.8	0.19
Leucine	180.8±8.55 (19)	24.7±7.06 (20)	156.1	0.33
Lysine	157.4±6.06 (19)	32.1±5.04 (20)	125.3	0.26
Methionine	31.6±1.41 (19)	13.1±1.81 (17)	18.5	0.04
Phenylalanine[2]	84.4±3.37 (20)	–	–	–
Threonine	111.3±4.51 (19)	19.3±5.64 (18)	92.0	0.19
Tryptophan	10.3±0.33 (19)	2.6±0.43 (18)	7.7	0.02
Valine	128.3±6.0 (19)	22.6±4.20 (19)	105.7	0.22

Values are means ± S.E.M. (N).
[1]The difference between the mean rates of protein synthesis in symbiotic and aposymbiotic aphids.
[2]The protein growth rate of aposymbiotic aphids on phenylalanine-free diet could not be quantified because of high mortality.

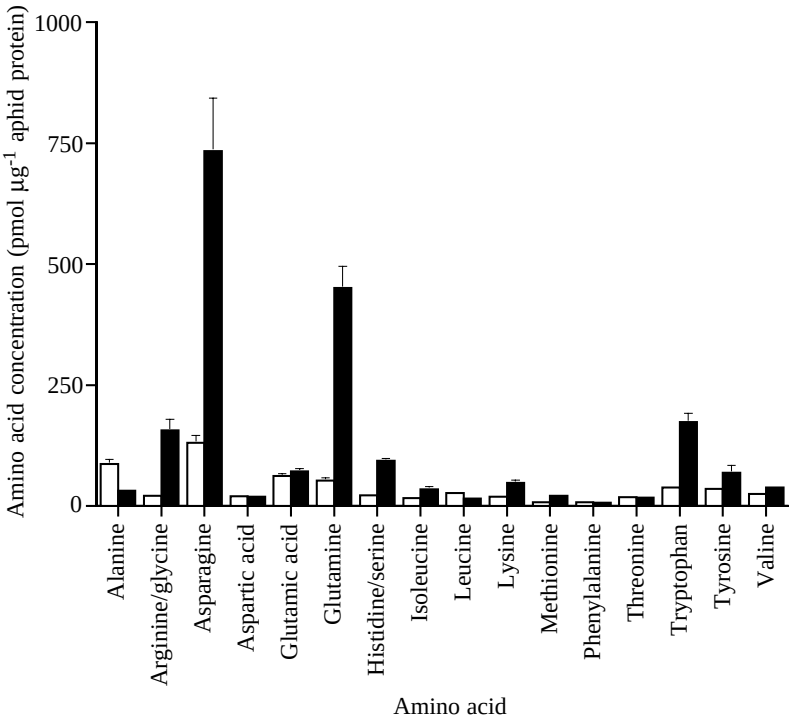

Fig. 2. Composition of amino acids in the free amino acid fraction of symbiotic (open columns) and aposymbiotic (shaded columns) *Aphis fabae* clone HR91/3. Values are means + S.E.M., *N*=9.

Table 3. *Ingestion and assimilation of dietary leucine (at 8.7 mmol l^{-1}) from the complete diet by 7- to 9-day-old symbiotic and aposymbiotic* Aphis fabae

Aphids	Diet ingested (µl aphid^{-1})	Leucine ingested (nmol aphid^{-1})	Leucine in honeydew (nmol aphid^{-1})	Leucine assimilated (%)
Symbiotic	1.00±0.141	8.7±1.23	0.53±0.080	94
Aposymbiotic	0.22±0.031	1.9±0.27	1.18±0.323	38

Values are means ± S.E.M. (*N*=12 replicates).

concentration of only one essential amino acid, leucine, was significantly higher in symbiotic aphids, at 27±2.7 pmol mg^{-1} protein, than in aposymbiotic aphids, at 16±1.3 pmol mg^{-1} protein (t_{16}=3.98, 0.01>*P*>0.001).

The depressed concentration of leucine in aposymbiotic aphids reared on the complete diet suggests that these aphids may have a reduced capacity to assimilate dietary leucine. To test this possibility, the assimilation of dietary leucine by 7-day-old symbiotic and aposymbiotic aphids reared from birth on the complete diet was compared (Table 3). On average, each symbiotic aphid ingested nearly four times more diet than aposymbiotic aphids, but the aposymbionts egested more leucine-derived radioactivity than the symbiotic aphids. From these data, it was calculated that symbiotic aphids assimilated more than 90 %, but aposymbionts assimilated less than 40 %, of the leucine they ingested. Of the leucine assimilated, 17 % (1.5±0.28 nmol leucine) was recovered from the protein fraction of symbiotic aphids and 2.6 % (0.05±0.011 nmol leucine) (means ± S.E.M., *N*=12) from the protein fraction of aposymbiotic aphids.

Impact of dietary amino acid omissions on Buchnera*-mediated amino acid biosynthesis*

The direct analysis of essential amino acid synthesis was conducted with [^{14}C]glutamic acid as precursor because, in initial experiments, *Aph. fabae* incorporated radioactivity from this amino acid into essential amino acids, but not from the other precursors tested ([^{14}C]alanine, [^{14}C]glycine or [^{14}C]serine). The amount of radioactivity recovered from [^{14}C]glutamic acid did not differ significantly between symbiotic and aposymbiotic aphids on a per unit protein basis (Table 4). After tissue fractionation, 20–30 % of the radioactivity was recovered from the low-molecular-mass fraction and protein hydrolysates. Relative to symbiotic aphids, the ^{14}C content of aposymbiotic aphids was significantly elevated for the low-molecular-mass fraction and significantly depressed for the protein hydrolysates (Table 4).

TLC analysis with autoradiography of the protein hydrolysates of symbiotic aphids yielded seven spots that were both radioactive and ninhydrin-positive: the non-essential amino acids alanine, aspartic acid+asparagine, glutamic

Table 4. *Incorporation of radioactivity from dietary [^{14}C]glutamic acid by symbiotic and aposymbiotic* Aphis fabae *on the complete diet*

Aphids	Carbon incorporated from dietary glutamic acid (nmol mg^{-1} aphid protein)		
	Total	Low-molecular-mass fraction	Protein hydrolysates
Symbiotic	1240±87 (6)	220±24 (6)	31±6 (6)
Aposymbiotic	1360±31 (6)	370±14 (6)	10±3 (4)
	t_{10}=1.39, P>0.05	t_{10}=5.64, P<0.001	t_8=2.69, 0.05>P>0.01

Statistical tests were conducted on logarithmically transformed data.
Values are means ± S.E.M. (N).

acid+glutamine and proline, and the essential amino acids isoleucine/leucine, lysine and threonine. HPLC separation with liquid scintillation counting confirmed these identifications (except that proline could not be detected by the HPLC method) and established definitively that all the radioactivity in the TLC isoleucine/leucine spot was exclusively in isoleucine. The ^{14}C-labelled amino acids in protein hydrolysates of aposymbiotic aphids were alanine, aspartic acid+asparagine, glutamic acid+glutamine and, as detected by TLC, proline. These data indicate that the *Buchnera* population in symbiotic aphids utilised carbon from dietary glutamic acid to synthesize the essential amino acids isoleucine, lysine and threonine.

Subsequent experiments explored the impact of omitting dietary isoleucine and threonine on [^{14}C]glutamic acid metabolism by the aphids. (The effects of omitting dietary lysine were not studied because the amount of radioactivity incorporated into lysine in aphids on the standard diet was close to the detection limit of HPLC with scintillation counting and too low to be quantified accurately.) The same arrays of ^{14}C-labelled amino acids were detected in aphids reared on the complete diet and on diets lacking isoleucine or threonine. Fig. 3 shows the amount of radioactivity in the five amino acids from protein hydrolysates of symbiotic aphids. On the complete diet, the greatest incorporation was into glutamic acid+glutamine, approximately three times greater than into aspartic acid+asparagine, isoleucine and threonine, and more than ten times greater than into alanine. The mean ^{14}C content of isoleucine and threonine was elevated in aphids on the diet lacking each of these amino acids, and the effect was statistically significant for isoleucine on the isoleucine-free diet. These results suggest that *Buchnera*-mediated isoleucine synthesis from dietary glutamic acid is increased by omitting isoleucine from the diet.

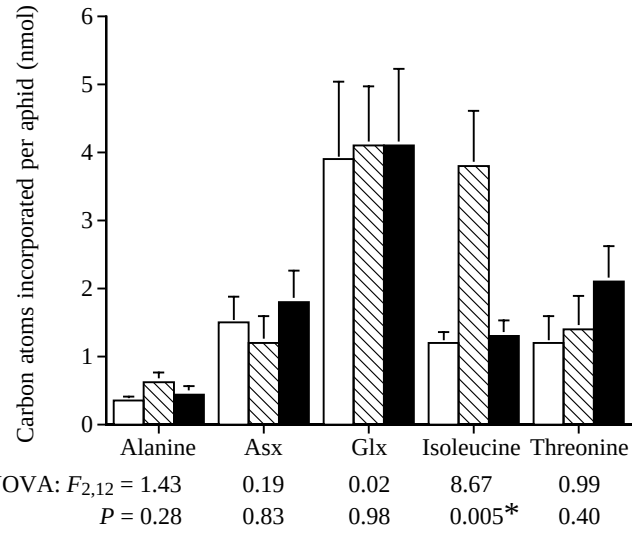

Fig. 3. Incorporation of radioactivity from dietary [U-^{14}C]glutamic acid into amino acids of protein hydrolysates from symbiotic aphids reared for 7 days on the complete diet (open columns), the isoleucine-free diet (hatched columns) and the threonine-free diet (filled columns). The critical value for significance of ANOVA tests after Bonferroni correction for five tests is P=0.01 (*significant). Values are means + S.E.M., N=5. Asx, asparagine plus aspartic acid; Glx, glutamine plus glutamic acid.

Discussion

The nutritional role of Buchnera *in* Aphis fabae

This study provides the first direct metabolic evidence for essential amino acid synthesis by *Buchnera* in *Aphis fabae*. Specifically, symbiotic but not aposymbiotic aphids incorporated radioactivity from dietary [^{14}C]glutamic acid into three essential amino acids, isoleucine, lysine and threonine, all of the aspartate biosynthetic family. These same three amino acids are synthesized from dietary glutamic acid by *Acyrthosiphon pisum* (Febvay et al., 1995), the only other aphid studied in this respect, pointing to some conservation of nutritional interactions in the aphid–*Buchnera* symbiosis between different aphid species. As proposed by Febvay et al. (1995) for *Ac. pisum*, glutamate assimilated from ingested diet by the aphid may be delivered to the *Buchnera* cells in the mycetocytes and transaminated to aspartate, from which these essential amino acids are derived. A further similarity between *Ac. pisum* and *Aph. fabae* is that radioactivity from dietary glutamic acid was not recovered from methionine, also an essential amino acid of the aspartate family (Febvay et al., 1995; this study), even though *de novo* methionine synthesis has been demonstrated for *Aph. fabae* fed on [^{35}S]sulphate (L. B. Minto, T. L. Wilkinson and A. E. Douglas, unpublished

results) and *Ac. pisum* fed on [^{14}C]sucrose (Febvay et al., 1999). Presumably, the carbon from dietary glutamate is incorporated into methionine at too low a rate to be detected in these experiments, but further research is required to establish the extent to which the carbon skeleton of methionine synthesized by the bacteria is derived from precursors other than dietary glutamate.

The complementary experiments exploring aphid performance on diets with individual amino acid omissions provide information on the significance of *Buchnera*-derived essential amino acids to *Aph. fabae*. In particular, the significantly reduced growth rates of symbiotic larvae on diets lacking methionine or histidine (Fig. 1) can be attributed to an insufficient supply of these amino acids from the symbiotic bacteria and preformed endogenous reserves. These data are in good accord with previous finding (Leckstein and Llewellyn, 1973) that a different *Aph. fabae* clone required methionine and histidine, but no other amino acid, for survival to adulthood. Leckstein and Llewellyn (1974) were able to exclude low feeding rates as the cause of poor aphid performance on these diets and, more generally, symbiosis-independent factors are unlikely to have contributed to the results because the growth rates of aposymbiotic *Aph. fabae* were not affected significantly by omission of either amino acid (Fig. 1). The failure of the symbiosis to meet the nutritional requirement of aphids for methionine and histidine over one insect generation is widespread, but not universal, among *Aph. fabae* clones; the performance of two of the eight *Aph. fabae* clones studied by T. L. Wilkinson and A. E. Douglas (in preparation) was not significantly reduced by dietary omission of these amino acids.

In the present study, the protein growth rates of symbiotic *Aph. fabae* on diets lacking each essential amino acid other than methionine and histidine did not differ significantly from the rates on the complete diet (Fig. 1), indicating that the aphid requirement for these amino acids over the 7-day experiment was met by endogenous sources, i.e. *de novo* synthesis by *Buchnera* and preformed reserves. The relative importance of reserves and *Buchnera*-mediated synthesis to the performance of symbiotic aphids on diets lacking essential amino acids other than phenylalanine cannot be discriminated definitively because the protein growth rates of aposymbionts on these diets were not depressed significantly relative to the complete diet. However, it can be concluded that *Buchnera*-mediated biosynthesis was crucial to the aphid requirement for phenylalanine because aposymbionts (which have the reserves but no *Buchnera*) suffered high mortality on the phenylalanine-free diet. Phenylalanine has long been suspected as 'the limiting amino acid in aposymbiotic aphids' (see Douglas and Prosser, 1991; Wilkinson and Ishikawa, 1999), and recent data highlighting abnormalities in the allosteric binding site of the enzyme prephenate dehydratase (Jiménez et al., 2000) suggest that regulation of phenylalanine synthesis in *Buchnera* may not be subject to feedback inhibition. In other words, *Buchnera* may be overproducing phenylalanine in the intact symbiosis.

These considerations raise a more general issue: the poor performance of aposymbiotic aphids on the complete diet. If, as argued in the Introduction and above, the principal role of *Buchnera* is to provide essential amino acids to the insect tissues, then the performance of aposymbiotic aphids on a diet containing all essential amino acids should match that of symbiotic aphids (Koch, 1956). Contrary to this expectation, the larval growth rates of aposymbionts of all aphid species studied on complete diets is invariably low, and either few survive to adulthood or larval development time is extended and the aposymbiotic adults produce few offspring that die without growing or developing. It has been suggested (Douglas, 1998) that the poor performance of aposymbionts may arise from limitations in their capacity to assimilate amino acids across the gut wall. The present study provides supportive evidence for one amino acid, leucine. The aposymbiotic *Aph. fabae* on the complete diet assimilated less than 40 % of ingested leucine and displayed a correlated reduction in both the absolute concentration of leucine in the free amino acid fraction (Fig. 2) and rates of protein synthesis relative to symbiotic aphids with both leucine or glutamate as precursor (Table 4). These data suggest that a shortfall of key essential amino acids, including leucine, which arises from the poor gut assimilatory capacity and absence of *Buchnera*-derived supply, limits aphid protein synthesis, with the consequent accumulation of other non-limiting amino acids in the free amino acid fraction. Aposymbiotic *Ac. pisum* on complete diets also exhibit elevated free amino acid concentrations (Prosser and Douglas, 1991; Liadouze et al., 1995), reduced rates of protein synthesis (Wilkinson and Douglas, 1996) and a depressed capacity to assimilate dietary essential amino acids, especially lysine (leucine was not studied) (Wilkinson and Ishikawa, 1999). Injection of essential amino acids into the haemocoel of aposymbiotic pea aphids also fails to alleviate the impact of the loss of symbiosis-derived essential amino acids (Wilkinson and Ishikawa, 2000), possibly because these injected amino acids are rapidly lost by respiration (T. L. Wilkinson, L. B. Minto and A. E. Douglas, in preparation). Further research is required to establish the relative importance of the absence of symbiosis-derived essential amino acids and the depressed gut assimilatory function in shaping the poor performance of the aposymbiotic aphids and to identify the biochemical basis of the impaired assimilatory capacity of aposymbiotic aphids.

The rates of essential amino acid synthesis by Buchnera

The dietary experiments in this study provide the first quantitative estimate of nutrient synthesis by the symbiotic micro-organisms in any insect. The production rates vary by more than an order of magnitude among the amino acids, from 0.02 fmol *Buchnera* cell^{-1} day^{-1} for histidine and tryptophan to 0.33 fmol cell^{-1} day^{-1} for leucine (Table 2). These rates refer explicitly to the contribution of *Buchnera*-derived amino acids to net protein growth, and they are less than the total amino acid synthesis rates because they do not take into account either

non-protein fates of amino acids, such as respiratory loss or as precursors of other compounds, or protein turnover.

The net amino acid production rates in Table 2 should not be treated as fixed characteristics of the symbiosis in *Aph. fabae*. The radiotracer experiments summarised in Fig. 3 indicate that the rate of bacterial synthesis of at least one essential amino acid, isoleucine, may vary with dietary supply, indicating that the contribution of bacterial-derived isoleucine to net protein growth is lower on diets containing isoleucine than the value of 0.19 fmol cell^{-1} day^{-1} obtained on isoleucine-free diet (Table 2). The greater impact of dietary deletion on isoleucine synthesis than on threonine synthesis is suggestive of differences in the regulation of net biosynthesis of these two amino acids. However, the regulatory mechanisms may be complex for, in plant-reared *Aph. fabae*, the synthesis of threonine varies more than that of isoleucine with rearing plant (T. L. Wilkinson, D. Adams, L. B. Minto and A. E. Douglas, in preparation). Although very little is known about the control of amino acid synthesis in the aphid symbiosis, these results for *Aph. fabae* are, in general terms, in good accord with other studies indicating that the rate of amino acid synthesis by *Buchnera* varies with diet composition in *Acyrthosiphon pisum* (Febvay et al., 1999) and with season for the sycamore aphid *Drepanosiphum platanoidis* (Douglas, 2000).

In summary, there is now growing evidence that the production rates of essential amino acids by *Buchnera* are not fixed, but may vary, possibly in direct response to changes in aphid demand, as influenced by the developmental stage, morph and growth rate of the aphid and the dietary supply of amino acids. This flexibility would promote sustained aphid performance on plants, the amino acid composition of which can vary with plant species and phenology, environmental conditions and even between different sieve elements of one plant (e.g. Winter et al., 1992; Douglas, 1993; Sandström and Pettersson, 1994; Blackmer and Byrne, 1999). Research priorities for the future are to establish the extent to which the biosynthetic rates of the various essential amino acids by the symbiotic bacteria may vary and to explore the underlying mechanisms.

We thank Dr Lynne Whitehead for comments on the manuscript and the Natural Environment Research Council for financial support.

References

Adams, D. and Douglas, A. E. (1997). How symbiotic bacteria influence plant utilisation by the polyphagous aphid, *Aphis fabae*. *Oecologica* **110**, 528–532.

Adams, D., Wilkinson, T. L. and Douglas, A. E. (1996). The aphid–bacterial symbiosis: a comparison between pea aphids (*Acyrthosiphon pisum*) and black bean aphids (*Aphis fabae*). *Ent. Exp. Appl*. **80**, 275–278.

Baumann, P., Baumann, L., Lai, C.-Y., Rouhbakhsh, D., Moran, N. A. and Clark, M. A. (1995). Genetics, physiology and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annu. Rev. Microbiol* **49**, 55–94.

Blackmer, J. L. and Byrne, D. N. (1999). Changes in the amino acids in *Cucumis melo* in relation to life-history traits and flight propensity of *Bemisia tabaci*. *Ent. Exp. Appl.* **93**, 29–40.

Buchner, P. (1966). *Endosymbiosis of Animals with Plant Microorganisms*. New York: John Wiley & Sons.

Charles, H. and Ishikawa, H. (1999). Physical and genetic map of the genome of *Buchnera*, the primary endosymbiont of the pea aphid *Acyrthosiphon pisum*. *J. Mol. Evol.* **48**, 142–50.

Douglas, A. E. (1988). Sulphate utilization in an aphid symbiosis. *Insect Biochem.* **18**, 599–605.

Douglas, A. E. (1989). Mycetocyte symbiosis in insects. *Biol. Rev.* **69**, 409–434.

Douglas, A. E. (1993). The nutritional quality of phloem sap utilized by natural aphid populations. *Ecol. Ent.* **18**, 31–38.

Douglas, A. E. (1998). Nutritional interactions in insect–microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu. Rev. Ent.* **43**, 17–37.

Douglas, A. E. (2000). Reproductive diapause and the bacterial symbiosis in the sycamore aphid *Drepanosiphum platanoidis*. *Ecol. Ent.* **25**, 256–261.

Douglas, A. E. and Prosser, W. A. (1992). Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pisum*) symbiosis. *J. Insect Physiol.* **38**, 565–568.

Febvay, G., Liadouze, I., Guillaud, J. and Bonnot, G. (1995). Analysis of energetic amino acid metabolism in *Acyrthosiphon pisum*: a multidimensional approach to amino acid metabolism in aphids. *Arch. Insect Biochem. Physiol.* **29**, 45–69.

Febvay, G., Rahbe, Y., Rynkiewiecz, M., Guillaud, J. and Bonnot, G. (1999). Fate of dietary sucrose and neosynthesis of amino acids in the pea aphid, *Acyrthosiphon pisum*, reared on different diets. *J. Exp. Biol.* **202**, 2639–2652.

Humphreys, N. J. and Douglas, A. E. (1997). The partitioning of symbiotic bacteria between generations of an insect: a quantitative study of *Buchnera* in the pea aphid (*Acyrthosiphon pisum*) reared at different temperatures. *Appl. Ent. Microbiol.* **63**, 3294–3296.

Jiménez, N., González-Candelas, F. and Silva, F. J. (2000). Prephenate dehydratase from the aphid endosymbiont (*Buchnera*) displays changes in the regulatory domain that suggests its desensitization to inhibition by phenylalanine. *J. Bacteriol.* **182**, 2967–2969.

Jones, B. N., Paabo, S. and Stein, S. (1981). Amino acid analysis and enzymatic sequence determination of peptides by an improved *o*-phthaldialdehyde precolumn labelling procedure. *J. Liquid Chromatogr.* **4**, 565–586.

Koch, A. (1956). The experimental elimination of symbionts and its consequences. *Exp. Parasit.* **5**, 40–97.

Leckstein, P. M. and Llewellyn, M. (1973). Effect of dietary amino acids on the size and alary polymorphism of *Aphis fabae*. *J. Insect Physiol.* **19**, 973–980.

Leckstein, P. M. and Llewellyn, M. (1974). The role of amino acids in diet intake and selection and the utilisation of dipeptides by *Aphis fabae*. *J. Insect Physiol.* **20**, 877–885.

Liadouze, I., Febvay, G., Guillaud, J. and Bonnot, G. (1995). Effect of diet on the free amino acid pools of symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*. *J. Insect Physiol.* **41**, 33–40.

Moran, N. A. and Baumann, P. (2000). Bacterial endosymbionts in animals. *Curr. Opin. Microbiol.* **3**, 270–275.

Morris, J. G. (1991). Nutrition. In *Environmental and Metabolic Animal Physiology* (ed. C. L. Prosser), pp. 231–276. New York: John Wiley & Sons.

Munson, M. A., Baumann, P. and Kinsey, M. G. (1991). *Buchnera*

gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte associated primary endosymbionts of aphids. *Int. J. Syst. Bacteriol.* **41**, 566–568.

Prosser, W. A. and Douglas, A. E. (1991). The aposymbiotic aphid: an analysis of chlortetracycline-treated pea aphid, *Acyrthosiphon pisum*. *J. Insect Physiol.* **37**, 713–719.

Prosser, W. A. and Douglas, A. E. (1992). A test of the hypotheses that nitrogen is upgraded and recycled in an aphid (*Acyrthosiphon pisum*) symbiosis. *J. Insect Physiol.* **38**, 93–99.

Rahbé, Y., Delobel, B., Febvay, G., Chategrel, B. (1994). Aphid-specific triglycerides in symbiotic and aposymbiotic *Acyrthosiphon pisum*. *Insect Biochem. Mol. Biol.* **24**, 95–101.

Raven, J. A. (1983). Phytophages of xylem and phloem – a comparison of animal and plant sap-feeders. *Adv. Ecol. Res.* **13**, 135–234.

Sandström, J. and Moran, N. (1999). How nutritionally imbalanced is phloem sap for aphids? *Ent. Exp. Appl.* **91**, 203–210.

Sandström, J. and Pettersson, J. (1994). Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrthosiphon pisum*) performance. *J. Insect Physiol.* **40**, 947–955.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407**, 81–86.

Vegotsky, A. and Fox, S. W. (1962). Protein molecules: intraspecific and interspecific variations. In *Comparative Biochemistry – A Comprehensive Treatise* (ed. M. Florkin and H. S. Mason), pp. 185–244. Amsterdam: Elsevier.

Wilkinson, T. L. (1998). The elimination of intracellular microorganisms from insects: an analysis of antibiotic treatment in the pea aphid (*Acyrthosiphon pisum*). *Comp. Biochem. Physiol.* **119**A, 871–881.

Wilkinson, T. L. and Douglas, A. E. (1996). The impact of aposymbiosis on amino acid metabolism of pea aphids (*Acyrthosiphon pisum*). *Ent. Exp. Appl.* **80**, 279–282.

Wilkinson, T. L. and Ishikawa, H. (1999). The assimilation and allocation of nutrients by symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum*. *Ent. Exp. Appl.* **91**, 195–201.

Wilkinson, T. L. and Ishikawa, H. (2000). Injection of essential amino acids substitutes for bacterial supply in aposymbiotic pea aphids *Acyrthosiphon pisum*. *Ent. Exp. Appl.* **94**, 85–91.

Winter, H., Lohaus, G. and Heldt, H. W. (1992). Phloem transport of amino acids in relation to their cytosolic levels in barley leaves. *Plant Physiol.* **99**, 996–1004.

Wright, J. P., Fisher, D. B. and Mittler, T. E. (1985). Measurement of aphid feeding rates on artificial diets using H-3-inulin. *Ent. Exp. Appl.* **37**, 9–11.