Appl. Entomol. Zool. 38 (2): 241–248 (2003)

High concentrations of trehalose in aphid hemolymph

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(Received 28 June 2002; Accepted 27 January 2003)

Abstract
The major components of hemolymph were examined by ^{1}H-NMR in seven species of aphids, *Acyrthosiphon pisum*, *Aphis gossypii*, *Aphis sambuci*, *Lachnus tropicalis*, *Megoura crassicauda*, *Myzus persicae* and *Uroleucon nigrotuberculatum.* Trehalose was detected as the main component from these aphids, and its concentrations in these insects were much higher (196 mM in *L. tropicalis* to 926 mM in *A. gossypii*) than those of two other homopterous insects, *Cryptotympana facialis* (91 mM) and *Nephotettix cincticeps* (53 mM). Concentrations in *L. tropicalis* and *A. gossypii* were equivalent to 1.6% to 2.0% of the fresh weight. Glucose appeared to be an artifact in the hemolymph, because it was not found in the hemolymph when validoxylamine A, a trehalase inhibitor, was added.

Key words: Aphids; homopterous; hemolymph; trehalose; ^{1}H-NMR

INTRODUCTION

Insects have high concentrations of trehalose in the hemolymph and they utilize the trehalose as an energy source in their tissues and organs by hydrolyzing it to two molecules of glucose (Wyatt, 1967). Hemolymph trehalose concentrations vary from 10 mM in *Bombyx mori* larva (Kono et al., 1993) to 255 mM in *Acyrthosiphon pisum* (Rhodes et al., 1997), depending on the species. Although studies on the regulation of hemolymph sugar levels are well established in many orders, the small size of a number of insect species makes it difficult to determine the hemolymph components. Aphids, plant phloem sap-feeding insects, have very high concentrations of sugars in their hemolymph (Ehrhardt, 1962; Wyatt, 1967; Rhodes et al., 1997) compared to other insect families. This suggests that high sugar concentrations in the hemolymph of aphids are associated with sugar concentrations in the phloem sap, the main nutritional source for aphids (Rhodes et al., 1997). However, information on hemolymph sugar levels in aphids is scarce, with the hemolymph sugar levels having been reported for only two species of aphids (Ehrhardt, 1962; Rhodes et al., 1997).

Currently, hemolymph components of insects are measured by ^{1}H-NMR (Thompson, 1990; Kono et al., 1993, 1994, 1995, 1999a, b; Takahashi et al., 1995; Phalaraksh et al., 1999; Lenz et al., 2001). NMR analysis has made it possible to determine the concentrations of sugars in a small sample and with minimal pretreatment. In this study, we investigated the major hemolymph components of seven species of aphids using ^{1}H-NMR, characterized the hemolymph of these insects and quantified the trehalose and other components.

MATERIALS AND METHODS

Insects. Five species of aphids, *Lachnus tropicalis*, *Uroleucon nigrotuberculatum*, *Megoura crassicauda*, *Acyrthosiphon pisum* and *Aphis sambuci* were collected from field colonies on host plants (Table 1) at Tsukuba City, Japan, from April to June 2001. *U. nigrotuberculatum* is an aphid of North American origin, which has inhabited the goldenrod, *Solidago altissima*, in Japan for approximately the past ten years (Ôtake, 1999). *Aphis gossypii* and *Myzus persicae* were collected from laboratory colonies maintained under 16L–8D at 20±0.5°C at the University of Tsukuba. Apterous adult females of each species were used for the collection of hemolymph. Male and female adults of

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Table 1. Profiles of aphid species used in this study

Aphid species	Host plants	Mean weight of adult (μg)[a]
Myzus persicae	*Brassica campestris*	685.2
Aphis gossypii	*Curcurbita moschata*	538.1
Aphis sambuci	*Sambucus racemosa*	714.5
Acyrthosiphon pisum	*Vicia fava*	2,277.8
Megoura crassicauda	*Vicia angustifolia*	2,514.4
Uroleucon nigrotuberculatum	*Solidago altissima*	3,313.3
Lachnus tropicalis	*Castanea crenata*	5,958.3

[a] $N=10$.

the cicada, *Cryptotympana facialis* (Homoptera: Cicadidae) collected from a field colony and female adults of the green rice leafhopper, *Nephotettix cincticeps* (Homoptera: Deltocephalidae) collected from laboratory colonies maintained with rice seedlings under 16L–8D at 25±0.5°C at the Takeda Chemical Industries, were used as references.

Collection of hemolymph and sample preparation. Apterous adult aphids and *N. cincticeps* were placed on double-sided Scotch® tape on a glass slide after CO_2 anesthesis, all legs were cut with forceps, and the exuding hemolymph was collected with 0.5 μl or 1 μl micro capillary tubes (EM minicaps, Hirschmann® laborgerate). Hemolymph obtained was immediately diluted with 500 μl of heavy water (D_2O) containing 0.1 ppm validoxylamine A (VAA), a specific inhibitor of trehalase (Asano et al., 1987; Kameda et al., 1987) and a small amount of phenylthiourea (an inhibitor of melanization). A total of 1 to 9 μl (*A. pisum*) of hemolymph was collected from 20 to 400 aphids. Ten microliter of hemolymph was collected from individual *C. facialis* through a small puncture on the dorsal part of the scutum. To investigate the effect of VAA on sugar degradation, two incubation experiments (25°C, 1 h) with and without VAA (0.1 ppm) were carried out. One experiment involved incubation of the diluted hemolymph of *A. pisum* (1 μl/500 μl D_2O) and the other, incubation of a homogenate supernatant of 5–7 female adults of *M. persicae* and *A. pisum* per each 500 μl of D_2O. All samples (hemolymph and homogenate) were centrifuged at 2,000×g for 10 min at 4°C. The supernatant (450 μl) was transferred to a tube with 50 μl of 1 mM sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP) as an internal standard and stored at −20°C until NMR analysis.

^{1}H-NMR analysis of the hemolymph. Hemolymph samples were subjected to ^{1}H-NMR (400 MHz) analysis with a JEOL JNM-EX 400 FT-NMR spectrometer. NMR spectra were obtained with the following parameters: a pulse width of 6.0 μs (45°), acquisition time of 2.048 s, pulse delay of 4.952 s, repetition time of 7.0 s and 64 or 128 pulses which acquired 32,768 data points covering a spectral width of 8,000 Hz at 21.8°C. The water signal at 4.8 ppm was suppressed by pre-saturation (homo-gated decoupling). Assignment of the signals of the chemicals on ^{1}H-NMR spectra was carried out by adding each standard chemical (2 mg) to the sample, and by checking the overlapping of the signals according to Kono et al. (1993). Quantification of the hemolymph components was estimated according to the calibration line between a standard chemical at a specific concentration and the relative signal strength of proton on each compound to that of TSP by ^{1}H-NMR, as previously described (Kono et al., 1995). For example, trehalose concentration in the hemolymph could be quantified by the following formula.

$$C_{\mathrm{Tre}}(\mathrm{mM})=(1/1.2)\times(S_{\mathrm{Tre1}}/S_{\mathrm{TSP}})\times((500+V)/V)$$

Where, C_{Tre} is the trehalose concentration in the hemolymph, (1/1.2) is a constant obtained from the calibration line of standard trehalose concentration. S_{Tre1} is the doublet peak height of trehalose at 5.2 ppm, S_{TSP} is TSP peak height (0.1 mM) at 0 ppm and V is the hemolymph volume (μl) in the samples. Trehalose content per aphid was also calculated from the homogenate samples. Numerals and letters in parentheses in Table 2 indicate the chemical shifts of the signals (δ unit) and the multiplicity of peaks: singlet (s), doublet (d), triplet (t), quartet

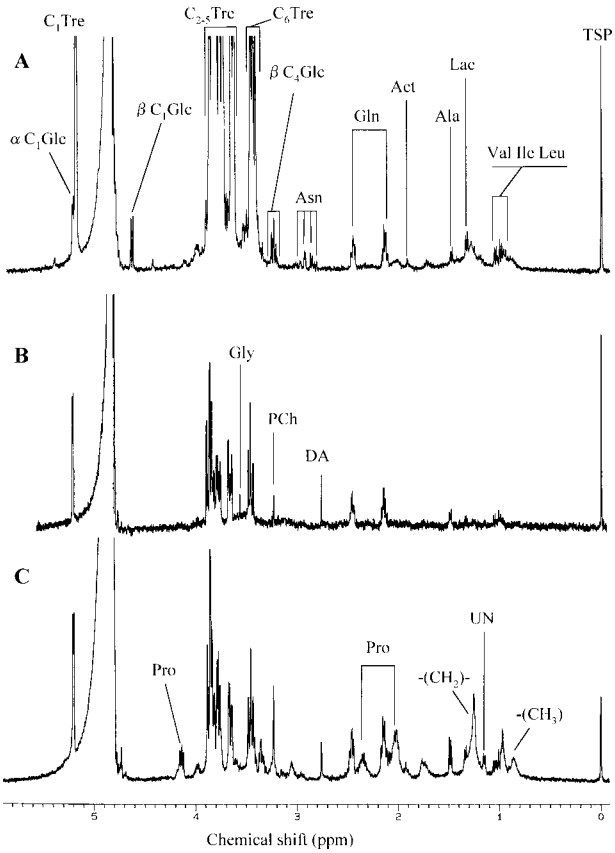

Fig. 1. ^{1}H-NMR spectra of the hemolymph from female adults of (A): *Acyrthosiphon pisum*, (B): *Cryptotympana facialis* and (C): *Nephotettix cincticeps.* Accumulation times of NMR pulsation in A, B and C as 64, 128 and 128, respectively. Hemolymph volumes were (A): 9 μl, (B): 10 μl, (C): 12 μl. TSP, sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (standard); -(CH_3), methyl moiety of fatty acid; Leu, leucine; Ile, isoleucine; Val, valine; -(CH_2)-, methylene moiety of fatty acid; Lac, lactic acid; Ala, alanine; Gln, glutamine; DA, dimethylamine; Asn, asparagines; PCh, phosphorylcholine; αGlc, α-glucose; βGlc, β-glucose; Tre, trehalose; Gly, glycine; UN, unknown.

(q) and multiplet (m).

RESULTS

Major components in the hemolymph of aphids compared to *C. facialis* and *N. cincticeps*

^{1}H-NMR spectra of hemolymph (9–12 μl hemolymph/500 μl D_2O) from female adults of *A. pisum*, *C. facialis* and *N. cincticeps* are shown in Fig. 1. Trehalose (5.20d) and glucose (α-CH_1 at 5.23d, β-CH_4 at 3.25t and β-CH_1 at 4.64d) were found in the hemolymph of *A. pisum.* Other components detected were leucine (0.96t), isoleucine

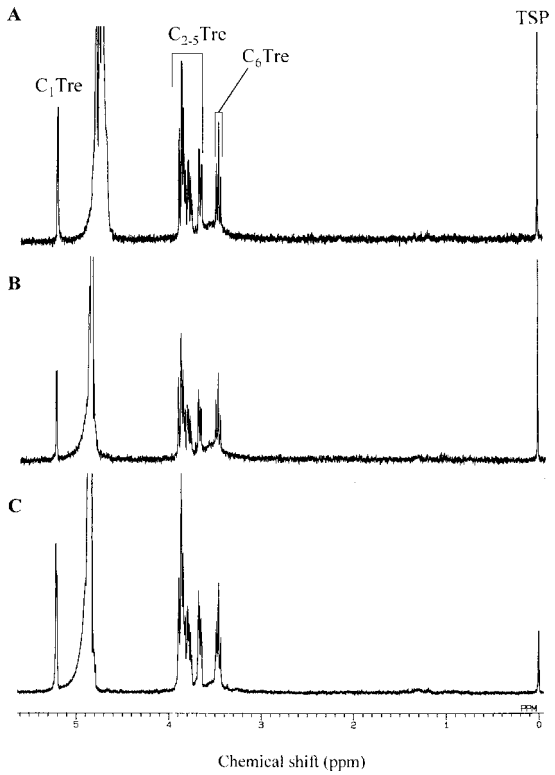

Fig. 2. ^{1}H-NMR spectra of the hemolymph from female adults of (A), *Lachnus tropicalis*; (B), *Aphis sambuci*; (C), *Aphis gossypii*, recorded with 128 pulses. Hemolymph volume, 1 μl. TSP, see Fig. 1; Tre, trehalose.

(1.02d), valine (1.00d, 1.05d), alanine (1.47d), glutamine (2.15q, 2.46q), asparagine (2.88q, 2.94q), lactic acid (1.33d), acetic acid (1.92s) and phosphorylcholine (3.24s). Trehalose was also found in *C. facialis* and *N. cincticeps*, but glucose was not found. ^{1}H-NMR spectra of 1 μl of hemolymph from apterous female adults of *L. tropicalis*, *A. sambuci* and *A. gossypii* are shown in Fig. 2. The trehalose signal could be detected even for small amounts of sample. Despite the high signal sensitivity for trehalose, the main sugars in phloem sap such as sucrose were not found. Trehalose was detected in the hemolymph of all the aphids examined, but glucose was rarely detected. Concentrations of hemolymph components of the three species of homopterous insects are shown in Table 2. Trehalose concentration in *A. pisum* (271.3 mM, N=5) was about three times higher than that of *C. facialis* (90.5 mM, N=2) and five times higher than that of *N. cincticeps* (52.6 mM, N=3). Glutamine was the most abundant amino acid in the three species and its concentration in *C. facialis* (48.4

Table 2. Quantification of hemolymph components in female adults of *A. pisum*, *Cryptotynpana facialis* and *Nephotettix cincticeps* by ^{1}H-NMR[a]

Chemicals	Concentration in mM			
	Chemical shift[b]	*A. pisum*	*C. facialis*	*N. cincticeps*
Leucine	0.964 t	1.4	ND	4.2
Isoleucine	1.022 d	2.7	ND	3.2
Valine	1.054 d	4.1	1.0	5.1
Lactate	1.327 d	4.5	5.4	8.8
Alanine	1.473 d	2.0	5.2	18.9
Acetate	1.924 s	0.6	ND	2.7
Proline	2.009 m	ND[c]	ND	8.4
Glutamine	2.455 q	19.2	48.4	65.5
Dimethylamine	2.754 s	0.3	1.7	2.9
Asparagine	2.882 m	15.7	ND	ND
Phosphorylcholine	3.223 s	0.2	0.6	6.5
Glycine	3.565 s	ND	1.6	ND
Glucose	4.657 d	17.7	ND	ND
Trehalose	5.206 d	271.3±4.2[d]	90.5	52.6

[a] Values are expressed as mean (N=3 in *N. cincticeps*, N=2 in *A. pisum* and *C. facialis*).
[b] Multiplicity of peaks, s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet.
[c] ND: Not detected.
[d] N=5 (mean±SD).

Table 3. Comparison of trehalose concentration in aphids

Species	Stage[a]	Trehalose (mM)	No. samples	Reference
A. pisum	A, ♀	271.3±4.2[bc]	5	this study
	A, ♀	255.0		Rhodes et al. (1997)[d]
A. gossypii	A, ♀	925.5	3	this study
A. sambuci	A, ♀	315.8	2	this study
L. tropicalis	A, ♀	196.1	3	this study
M. crassicauda	A, ♀	215.5±11.7[c]	5	this study
Megoura visiae	A, ♀	161–197		Ehrhardt (1962)[d]
M. persicae	A, ♀	678.9±170.3[c]	5	this study
U. nigrotuberculatum	A, ♀	313.8	3	this study

[a] A: apterous adult.
[b] The data shown in Table 2.
[c] Values expressed as mean±SD (N=5).
[d] Data obtained by paper chromatography.

mM) and *N. cincticeps* (65.5 mM) was much higher than that of *A. pisum* (19.2 mM). Asparagine was the second most abundant in *A. pisum* (15.7 mM) but it was not found in *C. facialis* and *N. cincticeps*. Overall, the concentrations of amino acids, organic acids and other components (dimethylamine and phosphorylcholine) in the hemolymph of *A. pisum* detected by ^{1}H-NMR were lower than those of *C. facialis* and *N. cincticeps*.

Trehalose concentrations in aphids

Trehalose concentrations in the seven aphid species are shown in Table 3. The concentration of 196.1 mM in *L. tropicalis* (N=3) was the lowest of the seven aphid species. Average trehalose concentrations in the hemolymph of *M. crassicauda* (N=5), *A. sambuci* (N=2) and *U. nigrotuberculatum* (N=3) were 215.5 mM, 315.8 mM and 313.5 mM, respectively. Those of *M. persicae* (N=5) and *A.*

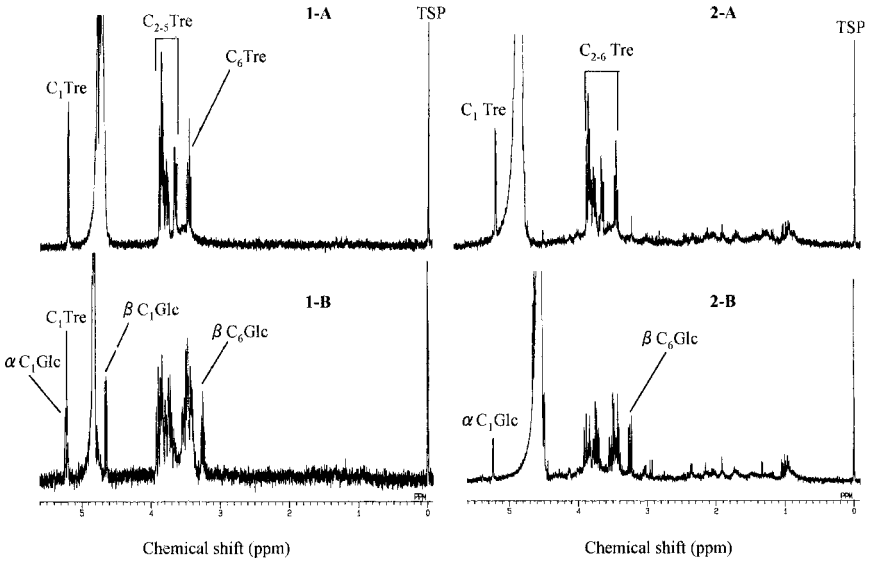

Fig. 3. ^{1}H-NMR spectra of the hemolymph of *A. pisum* (1) and the homogenates of *Myzus persicae* (2), with VAA (A) and without VAA (B). Accumulation of NMR pulsation is (1-B), 64; (1-A, 2-A and B), 128. Hemolymph volume, 1 μl (1-A and B). Total fresh weight, (2-A), 5 mg; (2-B), 6.2 mg. TSP, see Fig. 1; αGlc, α-glucose; βGlc, β-glucose; Tre, trehalose.

gossypii (N=3) were very high, 678.9 mM and 925.5 mM, respectively. However, trehalose concentrations varied from 459.5 mM to 879.8 mM in *M. persicae.*

Trehalose degradation in the hemolymph and homogenates of aphids

^{1}H-NMR spectra of hemolymph from apterous female adults of *A. pisum* (N=3) with and without VAA are shown in Fig. 3. Glucose (493.9 mM) and trehalose (43.3 mM) were present in the samples without VAA, whereas much trehalose (266.2 mM) and no glucose were found in the samples with VAA (1-A and B). In ^{1}H-NMR spectra of homogenates of apterous female adult *M. persicae*, a large amount of glucose (2.7±0.5 mg/100 mg insect) (N=5) was found in the homogenate sample without VAA, whereas trehalose was detected as a main component in the homogenate sample with VAA (2-A and B). Trehalose contents in the body were calculated to be 1.6±0.2 mg/100 mg insect (N=5) in *M. persicae* and 2.0±0.2 mg/100 mg insect (N=3) in *A. pisum.*

DISCUSSION

Numerous analytical studies have been conducted on the hemolymph composition of various insects, and concentrations of sugars, amino acids, fatty acids and organic acids have been quantified (Chen, 1985; Mullins, 1985; Sasaki and Ishikawa, 1995). In this study, ^{1}H-NMR spectroscopy revealed the presence of trehalose, amino acids and other components in the hemolymph of aphids and other homopterous insects (Fig. 1). Despite the minute quantities of hemolymph (as low as 0.25 μl), ^{1}H-NMR spectroscopy was sufficient to quantify the trehalose concentration. Therefore, this technique made it possible to examine trehalose metabolism in aphids and other homopterous insects.

Trehalose was present as a main component in the hemolymph of aphids (Figs. 1 and 2). Ehrhardt (1962) reported that sugar components in the hemolymph of *Megoura viciae* are comprised of trehalose, glucose, and small amounts of sucrose and fructose. Rhodes et al. (1997) reported that the presence of a high concentrations of trehalose (255 mM) and fructose (129 mM) in the hemolymph of *A. pisum* fed on an artificial diet containing 730 mM sucrose. In aphids, fructose degraded from dietary sucrose in the gut is absorbed into the hemolymph more efficiently than glucose, which is utilized for oligosaccharide synthesis in the gut (Ashfold et al., 2000). Thus, it is possible that some fructose is present in aphid hemolymph. In the present study, however, fructose and sucrose were not found in the hemolymph of aphids. It is

known that insects immediately synthesize monosaccharides, such as glucose and fructose, to trehalose in the fat body (Wyatt, 1967; Kono et al., 1998). The finding that no fructose or sucrose was found in the hemolymph of aphids suggests that fructose absorbed into the hemolymph from the gut of aphids is immediately converted to trehalose, and that this trehalose is the main blood sugar in aphids as well as in other insects.

Trehalose concentrations in the aphids studied were much higher (over 200 mM) than those of *C. facialis* (90.5 mM) and *N. cincepcis* (52.6 mM), while the concentrations of amino acids, organic acids and other components detected commonly from these insects by ^{1}H-NMR were the same (Tables 2 and 3). In particular, *M. persicae* and *A. gossypii* accumulated extraordinarily high levels of trehalose in their hemolymph. In most of the hemolymph sugars studied in insects, trehalose concentrations are regulated at levels from 10 to 50 mM, and few are known to exceed 100 mM (Wyatt, 1967). In the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae), trehalose concentrations in the hemolymph were recorded to be 181 mM under solitary conditions and 76 mM under gregarious conditions (Lenz et al., 2001). In the pupa of the worker honey bee, *Apis mellifera* (Hymenoptera: Apidae), a trehalose concentration of 123 mM was recorded (Itoh et al., unpublished). Hence, hemolymph trehalose concentrations of *M. persicae* and *A. gossypii* in this study appear to be the highest thus for reported. In addition, *M. persicae* (Fig. 3, 2-A) and *A. pisum* also contain 1.6% and 2.0% trehalose, respectively. It is known that certain insects accumulate a large amount of trehalose during diapause. The overwintering prepupa of the sawfly, *Trichiocampus populi*, contains 5 to 8% trehalose. This is the highest amount of trehalose reported from an insect species (Asahina and Tanno, 1964). Trehalose levels in winter diapausing pupa of the onion maggot, *Delia antiqua* reach about 1.5% (Nomura and Ishikawa, 2001). Trehalose concentrations in the aphids reported here are comparable to that of *D. antiqua.* These data confirm that a large amount of trehalose is generally contained in aphid hemolymph under normal conditions.

The diversity of trehalose concentrations between aphids and other homopterous insects is thought to be due to differences in carbohydrate requirements, because these insects feed on different plant tissues. Phloem sap is an important nutritional source for aphids. Its sugar components consist of up to 30% sucrose, with a small amount of galactose and sugar alcohols (Ziegler, 1975; Zimmermann and Ziegler, 1975). *N. cincticeps* feeds on xylem sap as well as phloem sap of the rice plant, *Oryza sativa* (Kawabe et al., 1980). Most cicadas like *C. facialis* are known to feed on xylem sap of wood. The only carbohydrate present in phloem sap of rice is sucrose (Kawabe et al., 1980). Therefore, hemolymph trehalose concentrations in aphids appear to directly reflect the concentration of sucrose in the phloem sap of their host plants.

A large amount of glucose was detected in hemolymph and homogenate samples when VAA was not added to the samples (Fig. 3). Glucose concentration in the hemolymph was reported to be higher than that of trehalose in *Manduca sexta* (Phalaraksh et al., 1999). However, trehalose was detected as a main component in the homogenate and hemolymph samples containing VAA. It is very probable that trehalose was hydrolyzed to glucose by the trehalase remaining in the samples. Though there are many reports on carbohydrate levels in insect hemolymph showing the presence of high concentrations of glucose as well as trehalose, glucose contents need to be reassessed using samples prepared without trehalase activity. We recommend the supplement of trehalase inhibitors as VAA during sample preparation for the analysis of sugar content of insect samples.

As for other hemolymph components of aphids, two non-essential amino acids, glutamine and asparagine were found in the hemolymph of *A. pisum* (Fig. 1). In contrast, asparagine was not detected in *C. fasialis* and *N. cincticeps*, while these insects have very high concentrations of glutamine in their hemolymph. Glutamine and asparagine are the main amino acids in the hemolymph of *A. pisum* (Sasaki and Ishikawa, 1995), and also in the honeydew and phloem sap of *Vicia fava*, the host plant of *A. pisum* (Sasaki et al., 1990). These two amino acids are converted to glutamic acid and aspartic acid by bacteriocytes, specialized cells in the fat body of aphids, and play important roles as a nitrogen source in the synthesis of essential amino acids by the bacterial symbiont, *Buchnera* (Sasaki and Ishikawa, 1995; Shigenobu et al., 2000). ^{1}H-NMR

spectroscopy makes it possible to examine amino acid metabolism. However, hemolymph volumes collected from each aphid were estimated to be from 3 nl (*A. gossypii*) to 180 nl (*L. tropiculis*) in this study (data not shown). It appears that a volume of more than 10 μl of hemolymph is required for ^{1}H-NMR analysis to investigate the metabolism of amino acids.

ACKNOWLEDGEMENTS

We are grateful to Dr. Yukihiko Kameda (University of Hokuriku) for providing VAA, Mr. Atsushi Iwata (Takeda Chemical Industries, Ltd.) for his gift of *N. cincticeps* and Mr. Yoichi Iwata and Mr. Yasuhiko Aiki (University of Tsukuba) for helping with the collection of aphids. Thanks are also due to Dr. DeMar Taylor (University of Tsukuba) for critical reading of the manuscript.

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