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Authors: Hongoh, Yuichi, and Ishikawa, Hajime

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Uric Acid as a Nitrogen Resource for the Brown Planthopper, *Nilaparvata lugens*: Studies with Synthetic Diets and Aposymbiotic Insects

Yuichi Hongoh and Hajime Ishikawa*

Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT—Planthopper harbors eukaryotic endosymbionts that are essential for normal development and growth of the host. Our previous studies suggested the possibility that the symbionts play an important role in nitrogen metabolism of the host through utilization of uric acid, a nitrogenous waste product. To examine the precise role of the symbiont, we prepared synthetic diets with various concentrations of amino acids, and measured uric acid contents stored in the insects reared on these diets. The results showed that planthopper synthesizes uric acid not only as a waste product, but also as a storage product when it ingests an excess amount of amino acids. We also investigated effects of the uric acid storage on growth of the normal and symbiont-depleted host. It turned out that in nitrogen deficiency the stored uric acid is consumed by the symbiont in order to sustain the growth of the host. In addition, we noted that the uric acid content of the host egg is highest at oviposition, and decreases significantly with its development, suggesting that the egg is supplied with uric acid by its parent prior to oviposition. These results are reminiscent of the nitrogen recycling reported for cockroaches.

INTRODUCTION

Most homopterans feed only on plant saps, which are generally poor in nitrogenous compounds with very unbalanced amino acid compositions (Mittler, 1958; Weatherley *et al.*, 1959; Auclair, 1963; Barlow and Randolph, 1978; Fukumorita and Chino, 1982; Sasaki *et al.*, 1990). Deficiency in nitrogenous compounds, that in essential amino acids in particular, is critical to all animals. Endosymbionts which most homopterans harbor have often been suggested as playing important roles to solve this problem (Buchner, 1965; Houk and Griffiths,1980). In aphid, prokaryotic endosymbionts are thought to supply amino acids, vitamins and other nitrogenous materials to their hosts (Ehrhardt, 1968; Mittler, 1971; Houk and Griffiths, 1980; Douglas, 1988; Douglas and Prosser, 1992), and it has been suggested that the symbionts play a key role in the host's nitrogen recycling utilizing glutamine that is otherwise a major nitrogenous waste product of aphid (Sasaki and Ishikawa, 1993, 1995; Febvay *et al.*, 1995). Planthoppers harbor eukaryotic yeast-like endosymbionts (Buchner, 1965; Chen *et al.*, 1981a). They produce uric acid which researchers have often failed to detect in either aphid body or honeydew, its excrements (Truskowski and Chajkinowna, 1935; Mittler, 1958; Lamb, 1959; Sasaki and Ishikawa, 1990; Hongoh and Ishikawa, unpublished data, 1996), which suggested that nitrogen metabolism of planthopper differs considerably from that of aphid. Actually, circumstantial evidence suggested that planthopper, with the aid of its endosymbiont, utilizes uric acid as a nitrogen resource. Sasaki *et al.* (1996) demonstrated that the whole body content of uric acid of symbiont-depleted, or aposymbiotic, planthoppers is significantly higher than that of the symbiotic insects, and that the uricase activity is only detected in isolated symbionts, but not in the tissues of aposymbiotic insects. A utilization of uric acid and the symbiont's role in it have already been reported for cockroaches and termites that are phylogenetically distant from planthopper. In the termite, *Reticulitermes flavipes*, it was shown that three species of bacteria in the hindgut are capable of an anaerobic degradation of uric acid to the classical products of bacterial fermentation including ammonia, and that the termite assimilates ^{15}N from ingested ^{15}N-uric acids in spite of its lacking uricase (Vogels and Van Der Drift, 1976; Potrikus and Breznak, 1977, 1981). Cockroaches are known to store uric acid, and it was suggested that the symbionts harbored in mycetocyte adjacent to urate cells, specialized cells for uric acid storage, convert it into a usable form for the host (Cochran, 1985).

In this study, we further investigated nitrogen metabolism of the brown planthopper, *Nilaparvata lugens*, employing synthetic diets and aposymbiotic insects. Results evidenced that this insect utilizes the uric acid storage as a nitrogen resource with the aid of its endosymbionts in a manner similar to that in cockroaches.

* Corresponding author: Tel. +81-3-5800-3553; FAX. +81-3-5800-3553.

MATERIALS AND METHODS

Insects

Brown planthoppers, *Nilaparvata lugens*, were maintained on rice seedlings at 25°C under 16 hr light: 8 hr dark photoperiodic regime. In experiments with synthetic diets, newly hatched insects within 24 hr (0 day old) were transferred to cylindrical plastic cases (6 cm diam., 5 cm high) with moistured filter papers and parafilm-wrapped synthetic diets attached on the both sides. About 50 insects were reared in a single case. Insects feeding on the diets attained adulthood between day 15 and 23 after hatch, depending on the concentration of the total amino acids contained by the diets. We used the short winged morph exclusively throughout the present study.

To eliminate the yeast-like symbionts, 0 day old nymphs were exposed to 35°C for 36 hr (Noda and Saito, 1979; Chen *et al.*, 1981b; Sasaki *et al.*, 1996). After the heat-treatment, insects were kept at 25°C as control insects.

Eggs of planthoppers were obtained by the method described by Sekido and Sogawa (1976). Planthopper eggs laid into the solution containing 5% sucrose and 0.004 M salicylic acid were collected and kept in distilled water at 25°C. The eggs hatched 8-9 days after oviposition under these conditions.

Synthetic diets

The synthetic diet was prepared according to Mitsuhashi and Koyama (1971) with a slight modification (Table 1). When used in experiments, total amino acid concentrations were varied between 0 and 500 mM without any change in the other compositions.

Determination of uric acid content

Uric acid content in whole tissues of planthopper was determined using uricase as described by Valovage and Brooks (1979) and Sasaki *et al.* (1996). Uric acid in honeydew was detected by reverse phase HPLC (Sasaki *et al.*, 1991). We were able to identify uric acid and determine its content at a retention time of 3.50 ± 0.02 min at a flow rate of 0.8 ml/min.

Statistic analysis

Individual data were obtained using tens of planthoppers, and summaries were expressed in the tables and figures as means ± SD of the several individual data. Relative growth rates (RGR) were calculated from fresh body weight (w) of planthoppers on the formula: RGR = (1/W)dw/dt. Data were analyzed using t-test, with a significant level being 5% in case there is no indication.

RESULTS

Accumulation of uric acid in the whole body

Planthoppers were reared from day 0 after hatch on the synthetic diets. Their uric acid content of the whole tissues on day 14, and their relative growth rate between day 4 and 14 were determined and expressed in relation to the concentration of total amino acids contained by the diet used (Fig. 1). The relative growth rate of insects was not significantly changed when the amino acid concentration was over 200 mM. In contrast, the amount of uric acid accumulated in their whole tissues was increased depending on the amino acid concentration, even when over 200 mM, and it attained four times as much at 500 mM as at 200 mM. No uric acid excretion from these insects was detected throughout the experiment.

Table 1. Composition of synthetic diet

(a) Vitamins, metals and other components

Vitamins	mg/l	Salts	mg/l
Thiamin HCl	25	$MgCl_2$	2000
Riboflavin	50	KH_2PO_4	5000
Nicotinic acid	100	$FeCl_3 \cdot 6H_2O$	20
Pyridoxin HCl	25	$CuCl_2 \cdot 2H_2O$	3.0
Folic acid	2.0	$MnCl_2 \cdot 4H_2O$	8.0
Ca pantothenate	50	$ZnCl_2$	4.0
Inositol	500	$CaCl_2 \cdot 2H_2O$	30
Cholin Cl	500		
Biotin	1.0	Sucrose	50000
Ascorbic acid	900	Cholesteryl benzoate	5.0

(b) Amino acids for 500 mM diet*

Amino acids	mM	Amino acids	mM
Alanine	25	Leucine	25
Arginine ·HCl	25	Lysine ·HCl	25
Aspargine	50	Methionine	20
Aspartic acid	25	Phenylalanine	25
Cysteine $\cdot 2H_2O \cdot$HCl	10	Proline	25
Glutamine	50	dl-Serine	25
Glutamic acid	25	Threonine	25
Glycine	25	Tryptophan	18
Histidine	25	Tyrosine	2
Isoleucine	25	Valine	25

*Synthetic diets containing other concentrations of amino acids were prepared by diluting this mixture, the other component shown in (a) being kept constant.

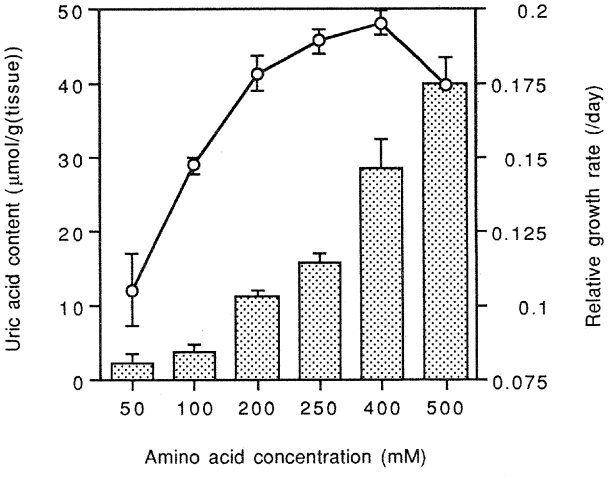

Fig. 1. Relative growth rate and uric acid content of whole tissues of planthopper in relation to the amino acid concentration contained by the synthetic diet. Insects were raised on day 0 after hatch on the synthetic diets containing various concentrations of amino acid as indicated. On day 4 and 14, they were weighed to estimate the relative growth rates during the period (open circles), and on day 14 their uric acid contents were determined enzymatically (columns). For each measurement, three distinct groups, each consisting of about 50 insects, were used. Values were expressed as means ± SD (n=3).

Changes in the uric acid content in symbiotic planthoppers

To further study relationship of the uric acid content of tissues to the amino acid concentration of the diet insects ingested, 0 day nymphs were raised on either 400 mM or 100 mM amino acid diet for 8 days. Subsequently, part of the nymphs were transferred to 50 mM diet, and further reared for 6 additional days. The rest of the nymphs were kept on the original diets for the same period. In each experiment, the uric acid content of the whole body tissues on day 8 and 14 were determined.

As shown in Fig. 2, in normal, or symbiotic, insects shifting the amino acid concentration of the diet from 400 mM down to 50 mM brought about an abrupt decrease of the uric acid content, while that of the insects kept on the 400 mM diet was steadily increased. When nymphs were raised on the 100 mM diet, their uric acid content was kept low throughout. Downshift of the amino acid concentration on day 8 further lowered its content.

Changes in the uric acid content in aposymbiotic planthoppers

In an effort to estimate roles played by endosymbionts, similar experiments as above were performed using aposymbiotic insects (Fig. 3). When aposymbiotic planthoppers were raised on the 400 mM diet, their uric acid content was about a half as much as that of symbiotic ones on the same diet both on day 8 and 14. This was mostly due to the difference of the body size between symbiotic and aposymbiotic insects, and the uric acid contents per unit weight of insect were much the same between the two (data not shown). Downshift of the amino acid concentration to 50 mM on day 8 stopped the uric acid increment in tissues, but did not cause such a sharp decrease in its content as observed with symbiotic insects.

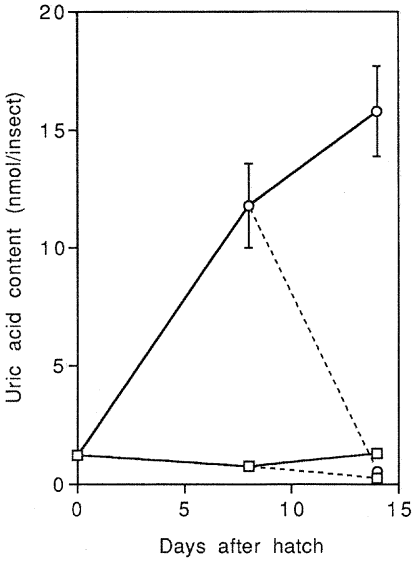

Fig. 2. Relationship between the amino acid concentration in the diet and the uric acid store in symbiotic planthoppers. About 50 nymphs were raised on synthetic diet containing 400 mM (○) or 100 mM (□) amino acids up to day 8, and a third of them were determined for their uric acid content. The rest were either kept on the same diets (solid lines) or transferred to synthetic diet containing 50 mM amino acids (broken lines), and further reared. On day 14, they were sacrificed for the determination of uric acid content. Based on the triplicated experiments, values are expressed as means ± SD.

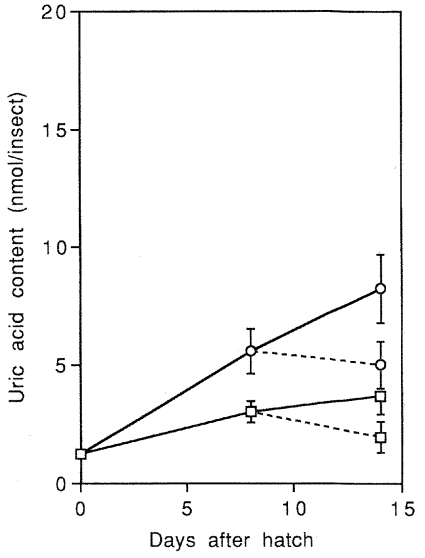

Fig. 3. Relationship between the amino acid concentration in the diet and the uric acid store in aposymbiotic planthoppers. In each experiment, about 50 newly-hatched nymphs were kept at 35°C for the first 36 hr to eliminate their symbionts, and raised as in Fig. 2 on synthetic diet containing 400 mM (○) or 100 mM (□). Values are expressed as means ± SD (n=3). Symbols are the same as those in Fig. 2.

On the 100 mM diet, the uric acid accumulation in aposymbiotic insects was more than in symbiotic ones. The high accumulation of uric acid in aposymbiotic insects was still more conspicuous when their smaller body size taken into consideration. Downshift of the amino acid concentration on day 8 incurred a significant decrease of the tissue uric acid content.

Effect of the diet on the insect growth rate

Planthopper nymphs were raised as above, and the effect of downshift of the amino acid concentration in the diet on their relative growth rate was determined (Table 2). In symbiotic planthoppers, whether raised on the 400 mM or 100 mM diet, downshift to 50 mM on day 8 led to a significant retardation of growth. The effect was more marked when amino acids were completely removed from the diet. In contrast, in aposymbiotic insects downshift of the amino acid concentration did not seem to be detrimental to the insect growth. Its shift from 400 mM down to 50 mM even seemed to profit the growth of aposymbiotic insects.

Uric acid excretion by aposymbiotic planthoppers

Uric acid was detected only in the honeydew from aposymbiotic planthoppers reared under the conditions examined, though it is possible that symbiotic insects also ex-

Table 2. Relative growth rate of planthoppers between 8 and14 days after hatch

Diet	Symbiotic	Aposymbiotic
100 mM	0.145 ± 0.004	0.073 ± 0.002
100 → 50 mM	0.091 ± 0.003	0.056 ± 0.005
100 → 0 mM	0.035 ± 0.000	–
400 mM	0.169 ± 0.006	0.024 ± 0.005
400 → 50 mM	0.130 ± 0.004	0.044 ± 0.008
400 → 0 mM	0.039 ± 0.000	–

About 50 newly-hatched nymphs were raised on synthetic diet containing 100 mM or 400 mM amino acids. For parts of insects, the amino acid concentration in the diet was shifted down to 50 mM or none on day 8. Insects were weighed day 8 and 14, and their relative growth rate per day was calculated. Values are expressed as means ± SD (n=3).

Table 3. Uric acid excretion by planthoppers on day 10 (nmol/insect/day)

Diet	Symbiotic	Aposymbiotic
100 mM	n.d.*	0.049 ± 0.010**
100 → 50 mM	n.d.*	0.036 ± 0.005**
400 mM	n.d.***	n.d.*
400 → 50 mM	n.d.*	0.049 ± 0.014**

Insects were reared as described under Table 2. Honeydew excreted by 10-50 insects was subjected to the determination of uric acid using HPLC. Detection limits were 0.035(*), 0.015(**) and 0.080(***) nmol/insect/day. Values are expressed as means ± SD (n=3). n.d., not detected.

creted uric acid under the limits of detection (Table 3). However, the amounts that might be excreted are too small to account for the decrease of the tissue content of uric acid observed in Fig. 2 when the amino acid concentration shifted down midway.

Presence of uric acid in planthopper egg

A considerable amount of uric acid was detected in planthopper eggs. The uric acid content was highest at the oviposition, and it gradually decreased as embryonic development progressed (Fig. 4). Its content of the embryo just before hatch (7-8 days after oviposition) was as low as 53% of that of the newly laid egg.

DISCUSSION

Although planthoppers are uricogenic just as many other insects, they normally do not excrete uric acid, but accumulate the compound in their tissues (Sasaki *et al.*, 1996). To estimate the relationship between their nitrogen ingestion and uric acid accumulation, we reared planthoppers on synthetic diets containing various concentrations of amino acids, and determined their uric acid content in tissues. As a result, it was found that their tissue content of uric acid increases depending on the amino acid concentration they ingest. In the meantime, it turned out that the insects' growth rate is no longer

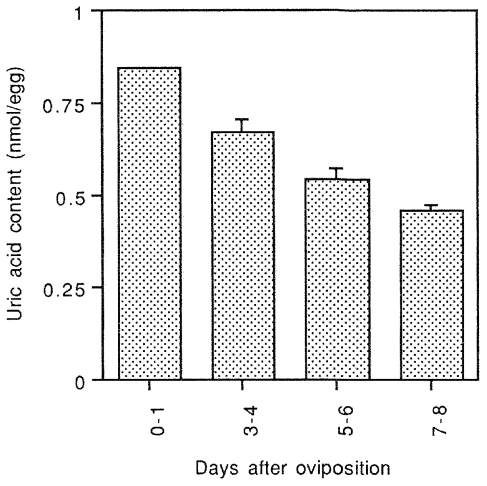

Fig. 4. Changes in uric acid content of planthopper during embryonic development. In each experiment, about 80 eggs that were laid by 5 parents were determined for the uric acid content. Values are expressed as means ± SD (n=5).

improved when the amino acid concentration in the diets is over 200 mM (Fig. 1). These results suggested that planthoppers synthesize uric acid not only as a nitrogenous waste product, but also as a storage product when they ingest an amount of nitrogen compounds more than they need.

That the uric acid store in planthopper tissues is used to make up for nitrogen compounds depleted from the diet was evident because downshift of the amino acid concentration in the diet caused a sharp decrease in the tissue content of uric acid (Fig. 2) without accompanying excretion of the compound (Table 3). In addition, deleterious effect of such the downshift on the insects' growth was significantly less when insects had stored more amount of uric acid beforehand by feeding on the diet containing more amino acids (Fig. 2, Table 2). These results suggested that planthoppers spare nitrogen compounds in the form of uric acid in provision for nitrogen deficiency they may encounter. This strategy is just reminiscent of cockroaches as described by Mullins and Cochran (1975). Cockroaches, when fed on excess amount of nitrogen compounds, will convert them into uric acid and store it in the urate cell as a nitrogen resource (Cochran, 1985). Nevertheless, downshifts of the amino acid concentration significantly retarded the insects' growth (Table 2), suggesting that no matter how much uric acid accumulated in the tissues, it cannot fully complement nitrogen deficiency in the diet.

Although aposymbiotic planthoppers feeding on the diet rich in amino acids accumulated uric acid in much the same way as symbiotic ones, unlike in the latter downshift of the amino acid concentration did not reduce the tissue content of uric acid significantly (Fig. 3). This is taken to suggest that endosymbionts play a pivotal role in planthopper's utilization of the uric acid store. As suggested previously (Sasaki *et al.*, 1996), the enzyme uricase present in the symbiont will initiate the reactions necessary for the uric acid utilization. In this context, it is intriguing that the amino acid shiftdown from 400

mM to 50 mM significantly improved the growth of aposymbiotic insects (Table 2). It is likely that for these insects the synthesis of a large amount of uric acid, which cannot be utilized, is nothing but a waste of energy. Uric acid excretion by aposymbiotic insects (Table 3) may be also related to their inability of utilizing uric acid. It is conceivable that the stored uric acid undergoes incessant dissolution, though little by little. While in symbiotic insects the dissolved uric acid will be used up instantaneously by the action of the symbiont's uricase, aposymbiotic insects, probably, cannot but excrete it, as it is, because of the lack of the enzyme activity.

In many uricotelic animals, embryogenesis takes place in a semi-closed space enclosed by the thick eggshell, and uric acid, a nitrogen waste product, tends to accumulate in the embryonic tissue as the development progresses. In contrast, in planthoppers the egg content of uric acid, which was highest at oviposition, gradually decreased with time (Fig. 4), suggesting that planthoppers provide uric acid for their egg as a nitrogen resource to sustain its embryonic development. Thus, it is likely that endosymbionts are not only transmitted to host's progeny through eggs, but also essential to their embryogenesis in utilizing the provided uric acid as a nitrogen resource. This, again, reminds us of cockroaches, which not only store uric acid for their own sake, but also provide the compound for their egg (Mullins and Keil, 1980). It is highly likely that in cockroaches the egg uric acid is utilized for embryogenesis because their endosymbionts are also transmitted through host's generations by way of eggs.

For phytophagous and detritophagous insects, to secure nitrogen resources should be a matter of paramount importance. It is well understood that one of the most efficient ways to do so is harboring endosymbionts with the ability of nitrogen recycling one way or another. Among ways of nitrogen recycling, the one that makes use of uric acid will be most accessible because the compound is a nitrogen waste product ubiquitous in insects. Probably for this reason, insect groups developed symbioses repeatedly with distinct microorganisms with the ability of mobilizing uric acid, such as yeast-like fungi in planthopper (Sasaki *et al.*, 1996) and bacteria in cockroach (Cochran, 1985) and termite (La Fage and Nuttings, 1978). As suggested in this work, exploiting the uric acid store is advantageous in that insects can transmit it as a resource to the next generation. However, the fact that production of uric acid costs much energetically might have induced some insects, such as aphids, to seek for an alternative, more efficient way of nitrogen recycling without producing uric acid (Sasaki and Ishikawa, 1995).

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