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Nitrogen Recycling in the Brown Planthopper, *Nilaparvata lugens*: Involvement of Yeast-like Endosymbionts in Uric Acid Metabolism

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Planthopper harbour yeast-like symbionts in the mycetocyte. A possible role that the symbionts may play for the host insect is nitrogen recycling, i.e. conversion of nitrogenous waste products of the insect into compounds of nutritional value. To examine this possibility, we determined the uric acid content in the honeydew and the whole tissues of control and symbiont-depleted brown planthoppers, *Nilaparvata lugens*. The symbiont-depleted insects were obtained by exposing newly hatched nymphs to 35°C for three days. In the honeydew, uric acid was not detected regardless of the presence or absence of symbionts. Nevertheless, a considerable amount of uric acid was found in the insect tissues, and the content was markedly higher in heat-treated insects than in controls. We also determined the uricase activity in the insect whole tissues and in the isolated symbionts. While in control insects uricase was detectable, there was no uricase activity in heat-treated insects. Isolated symbionts had uricase activity 15 times higher than in the whole tissues of control insects. These results suggested that uric acid synthesized by the insect is stored in insect tissues rather than excreted, and that uric acid is recycled in the planthopper with the aid of its endosymbionts.

Planthopper Yeast-like symbionts Uric acid Uricase Nitrogen recycling

INTRODUCTION

A number of plant-sucking insects possess obligatory endosymbionts (Buchner, 1965). In conjunction with the fact that plant sap is nutritionally poor, with a high carbon/nitrogen ratio and low levels of lipids and vitamins, the symbionts of sap feeders have often been suggested as playing important roles in the hosts' nutrition and metabolism (Houk and Griffiths, 1980). Planthoppers harbour yeast-like symbionts in the mycetocyte, a cell differentiated specifically for this purpose. The symbionts are transmitted directly from the maternal insect to her offspring by transovarial infection and are found in the host at every developmental stage (Chen *et al.*, 1981a). Although aposymbiotic planthoppers do not occur in nature, the symbionts can be disrupted by experimental methods such as exposure to high temperature or treatment with antibiotics (Chen *et al.*, 1981b).

The eggs laid by heat-treated brown planthoppers, *Nilaparvata lugens*, harbour only a few symbionts and are unable to hatch, suggesting that the symbionts are essential for normal embryonic development (Lee and Hou, 1987). In newly hatched nymphs of smaller brown planthoppers, *Laodelphax striatellus*, exposed to 35°C for three days, the population of the symbionts dramatically decreased and most of the nymphs failed to develop into adults (Noda and Saito, 1979). Analyses of sterols in control and heat-treated *L. striatellus* indicated that the symbionts provide the host insect with 24-methylenecholesterol (Noda *et al.*, 1979). It has also been proposed that the symbionts play significant roles in the host's nitrogen metabolism. Koyama (1985) investigated the amino acid requirement of *N. lugens* by maintaining newly hatched nymphs on synthetic diets from which individual amino acids were omitted, and reported that the insects were able to develop into adults in the absence of any one of the amino acids. It was postulated that the symbionts synthesized so-called essential amino acids for the host insect.

Nitrogen recycling, namely conversion of nitrogenous waste products of the host insect into compounds of nutritional value, is another possible role that the symbionts may play. So far, nitrogen recycling has been sug-

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gested for the three symbiotic systems; one is gut symbiosis, and the other two, intracellular symbioses. Termites and cockroaches are similar in that they deposit the uric acid they produce internally in specialized urate cells in the fat body. In the termite, *Reticulitermes flavipes*, it was shown that three species of bacteria in the hindgut are capable of the anaerobic degradation of uric acid to the classical products of fermentation including ammonia (Potrikus and Breznak, 1977). It was suggested that in cockroaches the uric acid store is converted into nitrogenous compounds of value by intracellular bacterial symbionts (Cochran, 1985). In contrast, the aphid does not produce uric acid (Sasaki *et al.*, 1990), but converts nitrogenous wastes into glutamine and asparagine, which, in turn, are used by its intracellular bacterial symbionts to reproduce essential nitrogenous compounds (Sasaki and Ishikawa, 1993, 1995).

In this study, we tested the possibility of nitrogen recycling in *N. lugens*, and suggest that this insect recycles nitrogenous compounds, utilizing its eukaryotic endosymbionts, in a way similar to those in termites and cockroaches.

MATERIALS AND METHODS

Insects

Brown planthoppers, *N. lugens*, were maintained on rice seedlings at 25°C under a 17 h light:7 h dark photoperiodic regime. While the population of planthoppers consists of short winged females, long winged females, short winged males and long winged males, we could tell neither morph nor sex before the insects underwent the final ecdysis. In the experiments where body weight and uric acid content of adult insects were determined, we used short winged morph.

To eliminate the yeast-like symbionts, nymphs newly hatched within 24 h (0 day old) were exposed to 35°C for three days according to the technique of Noda and Saito (1979) on *L. striatellus*. After the heat-treatment, the nymphs were kept at 25°C as control insects.

Histological procedures for light microscopy

The effect of heat-treatment on the symbiont population was examined by light microscopy. Fourth instar nymphs were decapitated and fixed in Carnoy's solution for 2 h. The fixed insects were dehydrated through an ethanol–xylene series, embedded in paraffin wax, and sectioned at 5 μm. The sections were dewaxed and hydrated through a xylene–ethanol–water series, and stained with Ehrlich's haematoxylin and the PAS reaction.

Determination of uric acid

Uric acid contents in the whole tissues, haemolymph and honeydew of control and heat-treated planthoppers were determined using uricase as described by Valovage and Brooks (1979). To determine uric acid from the whole tissues, insect materials (10–20 mg) were homogenized in 1 ml of 10 mM Li_2CO_3. The homogenate was incubated at 95°C for 10 min, and centrifuged at 10,000 ***g*** for 3 min. The supernatant was mixed with 5 vol of 10 mM Tris–HCl (pH 8.5), and the mixture was divided equally into two test tubes. One test tube received 150 μl of uricase solution at 0.5 u/ml (1 unit corresponds to the uricase activity that converts 1 μmol uric acid into allantoin/min at pH 8.5 at 25°C). The other received the same volume of distilled water. After incubation at room temperature for 30 min, the difference in absorbance at 292 nm between the two solutions was read in a spectrophotometer.

A sample of haemolymph (5–7 μl) was collected from 20-day-old planthoppers. The insect was anaesthetized in a stream of carbon dioxide and the fore legs were removed. By pressing the abdomen gently, haemolymph was collected with a capillary pipette. The haemolymph was mixed with 10 mM Li_2CO_3 to give a volume of 50 μl, and the uric acid content was determined as described above except that the volume of solutions was reduced.

To collect the honeydew excreted by planthoppers, 30 insects (20 days old) were kept on rice seedlings around which a plastic disc was placed. The honeydew droplets excreted onto the plastic disc during 24 h were washed off with distilled water. The samples were evaporated to dryness *in vacuo*, suspended in 50 μl of 10 mM Li_2CO_3, and assayed for uric acid as above. Data were analysed using the *t*-test with a significant level being 5%.

Isolation of yeast-like symbionts

The yeast-like symbionts were isolated basically according to the method of Noda and Omura (1992). Insect materials (approx. 0.5 g) were homogenized in 2 ml of a buffer consisting of 50 mM Tris–HCl (pH 7.0) and 250 mM sucrose. The homogenate was filtered through nylon mesh with a pore size of 90 μm. The filtrate was mixed with 90% Percoll containing the Tris–HCl buffer containing sucrose to give a final Percoll concentration of 30%, and centrifuged at 2,000 ***g*** for 10 min. The pellet was suspended in 1 ml of the same buffer, mixed with 5 ml of 90% Percoll, and the mixture was centrifuged at 80,000 ***g*** for 20 min. After centrifugation, the symbionts were recovered and washed once with the same buffer.

Uricase activity

Uricase activity in whole tissues and isolated symbionts was determined according to Osman *et al.* (1989). Insect materials (approx. 0.1 g) and isolated symbionts were crushed in a mortar chilled with liquid nitrogen and collected in a test tube containing 1 ml of 50 mM Tris–HCl (pH 8.0). The suspension was centrifuged at 40,000 ***g*** for 10 min. To the supernatant, protamine sulphate (final 0.2% w/v) was added to precipitate nucleic acids. After the precipitate was removed by centrifugation at 10,000 ***g*** for 5 min, ammonium sulphate was

added to the supernatant to give a final concentration of 60%. The solution was stirred for 30 min at 4°C and centrifuged at 10,000 *g* for 10 min. The precipitate was suspended in a minimal volume of 10 mM Tris–HCl (pH 8.0) and dialysed against the same buffer for 2 h at 4°C. The enzyme solution thus prepared was added into 10 vol of the reaction mixture [10 mM Tris–HCl (pH 8.0) containing 0.1 mM uric acid] and the decrease in the absorbance of the solution at 292 nm was recorded in a spectrophotometer. The protein content in the enzyme solution was determined using the BCA protein assay reagent (Piece Co.) with bovine serum albumin as a standard.

RESULTS

Effects of heat treatment on planthoppers and symbionts

Under the rearing conditions employed, more than 90% of control insects developed into adults. The final ecdysis occurred on about the 13th day after the hatch. When newly hatched nymphs were exposed to 35°C for three days and maintained at 25°C thereafter, they failed to attain adulthood although they survived as long as the control insects.

Figure 1 shows the changes of body weight of control and heat-treated insects. The body weight of control females rapidly increased after the final ecdysis and reached 3.1 mg on the 24th day of hatch. The body weight of males was almost constant at about 1 mg after the final ecdysis. Since heat-treated insects failed to attain adulthood, it was not feasible to distinguish between males and females, even on later days. They, as a mixture of males and females, showed a slow growth and their weight on the 24th day was only 1.5 mg.

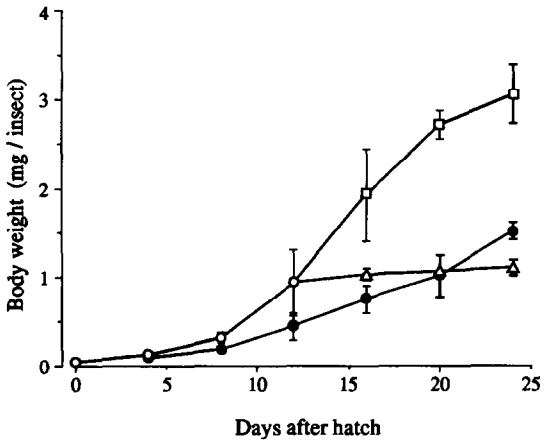

FIGURE 1. Changes in body weight of *N. lugens* during post-embryonic development. In each measurement, at least 10 insects were weighed in each group. In control insects, male and female were distinguishable only after the final ecdysis. For adults, only short winged males and females were weighed. In heat-treated insects, which failed to attain adulthood, neither sex nor morph was distinguishable. Values are expressed as means ± SD ($n = 3$). ○ Control insects (nymphs); □ control insects (adult females); △ control insects (adult males); ● heat-treated insects.

In the fat body of the fourth-instar nymphs of the control insects, a number of yeast-like symbionts were found which were distinctly stained by the PAS reaction [Fig. 2(A)]. In contrast, the symbionts were hardly detected in the fat body of the fourth-instar nymphs that had been heat-treated for three days at the newly hatched stage [Fig. 2(B)].

Uric acid content

Uric acid, a major nitrogenous waste of many insects, was not detected in either honeydew of control insects or heat-treated ones. Nevertheless, a considerable amount of uric acid did exist in the insect tissues. As shown in Fig. 3, during nymphal development of the control insects, the uric acid content varied between 8 and 23 nmol/mg tissues. In both female and male insects, the uric acid content slightly decreased after the final ecdysis and afterwards tended to increase. The uric acid content in heat-treated insects was markedly higher than in control insects.

Uric acid was also detected in the haemolymph of the planthoppers, and its concentration in 20-day-old insects was significantly higher in heat-treated than in control insects, which were 0.56 ± 0.17 and 0.22 ± 0.16 nmol/μl, respectively.

Uricase activity

The accumulation of uric acid in heat-treated insects suggested the involvement of symbionts in the utilization of uric acid. To examine this possibility, we determined the uricase activity in whole insects and isolated symbionts. Uricase catalyses the oxidization of uric acid into allantoin which is the first reaction in the breakdown of uric acid.

While in control insects the uricase activity was detectable, there was no activity in heat-treated ones. Also, it turned out that isolated symbionts contain 15 times more uricase activity than in the whole tissue of control insects, suggesting that the enzyme activity is located in the symbionts (Table 1).

DISCUSSION

In the present study, we demonstrated that (1) planthoppers do not excrete a detectable amount of uric acid; (2) the heat-treated insects contain more uric acid in their tissues than control insects (Fig. 3); and (3) the yeast-like symbionts have the uricase activity (Table 1). These results suggest that uric acid synthesized by the insect is stored in the insect's tissues rather than excreted as in many insects, and that uric acid is recycled in the planthopper with the aid of its symbionts. Maintaining the nymphs of *N. lugens* on synthetic diets, Koyama (1985) demonstrated that they are able to grow in the absence of any one of the essential amino acids. Taking these findings together, it is likely that symbionts synthesize essential amino acids for the host insect using uric acid as a nitrogenous source.

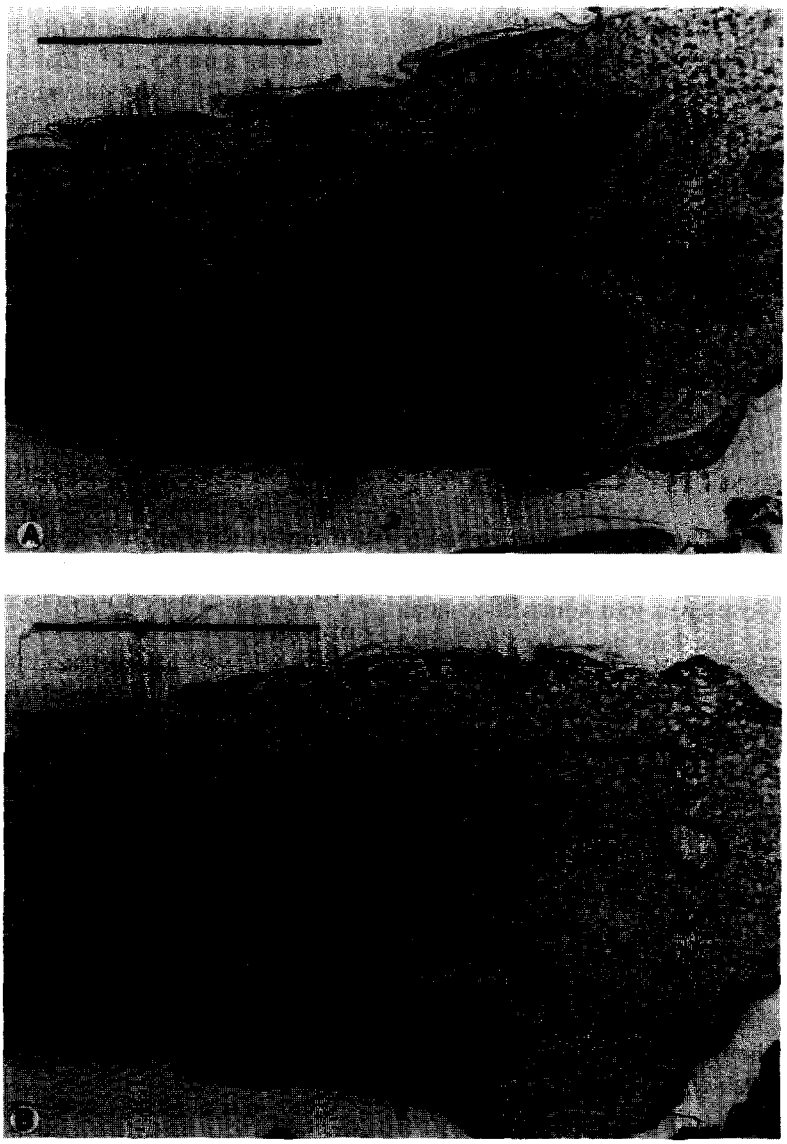

FIGURE 2. Abdominal vertical sections of the control (A) and heat-treated (B) 4th-instar nymphs stained with Ehrlich's haematoxylin and the PAS reaction. Yeast-like symbionts were stained distinctly with the PAS reaction (arrow). Go, gonad; Gu, gut; M, muscle. Scales represent 1 mm.

It is interesting that the planthopper seems to recycle nitrogenous wates in a way different from that in aphids, a close relative of the planthopper, which also feeds on plant sap. In the symbiotic system of an aphid, nitrogenous wastes are recycled via ammonia (Whitehead *et al.*, 1992) and amide amino acids such as glutamine and asparagine (Sasaki and Ishikawa, 1993, 1995), which is apparently a more efficient way of nitrogen recycling. This may be relevant to a notable difference in the mode of reproduction between these two insects. Planthoppers bear eggs while aphids produce progeny by viviparous parthenogenetic reproduction. Although it costs more to synthesize and re-utilize uric acid than to recycle it without producing uric acid, the planthopper synthesizes uric acid, possibly because it has to store nitrogenous wastes as an insoluble material during embryonic development within the egg shell. In fact, the uric acid content of control insects was highest just after hatching (Fig. 3). In this context, it will be interesting to discover the modes of nitrogen recycling in insects more closely related to aphids, such as scales and whiteflies.

Nitrogen recycling via uric acid in the planthopper instantly reminds us of that in the termite and cockroach. Although in both the termite and cockroach symbiotic bacteria seem to play a central role in mobilizing the uric acid store, there is one important difference. In the termite, the uric acid store in the fat body is mobilized by the gut symbionts of other individuals through necrophagy and/or cannibalism (La Fage and Nuttings, 1978). In contrast, it seems that in the cockroach the uric acid store in the urate cell is mobilized, as occasion demands, by the mycetocyte symbionts nearby in the fat body of the same individual (Cochran, 1976). In view of the close phylogenetic relationship between the termite and cockroach (Wilson, 1971), it is likely that their gut and mycetocyte bacteria share a common ancestor (Harada and

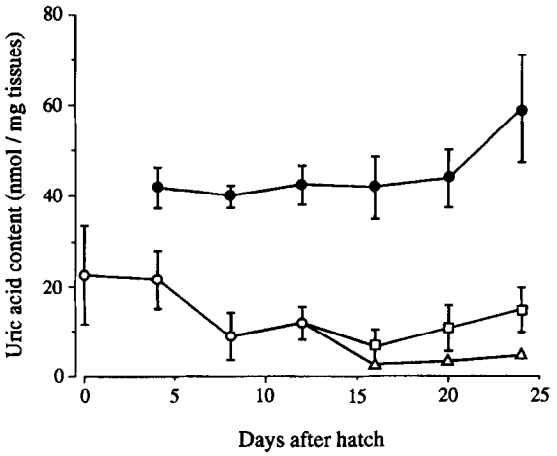

FIGURE 3. Changes in uric acid content of the insect whole tissues during post-embryonic development. In each experiment, uric acid was extracted from 10–20 mg of insect materials and determined spectrophotometrically using uricase. Values are expressed as means ± SD ($n = 3$). ○ Control insects (nymphs); □ control insects (adult, short-winged females); △ control insects (adult, short-winged males); ● heat-treated insects.

TABLE 1. Uricase activity in *N. lugens* and their yeast-like symbionts

	Units enzyme activity
Control insects	1.2
Heat-treated insects	—
Isolated symbionts	17.6

One unit of enzyme converts 1 nmol uric acid into allantoin/mg protein/min. Values are expressed as means of two independent runs. Uricase activity was not detected in heat-treated insects, the detection limit being 0.05 u.

Ishikawa, 1993). Nitrogen recycling in the planthopper is similar to that in the cockroach in that the individual makes use of its own uric acid store. However, the planthopper is phylogenetically distant from either the cockroach or termite. In addition, its mycetocyte symbiont is eukaryotic, different from that of cockroach. This may indicate that nitrogen recycling via uric acid in the planthopper has been acquired independently of those of the termite and cockroach.

So far, it has not been determined where the planthopper stores uric acid. Since the level of uric acid content in the haemolymph is higher in heat-treated insects than in control ones, it is possible that uric acid is stored in some special cell such as urate cells in the termite and cockroach. However, in respect of the fat body tissue of planthopper examined under a light microscope, no special cells other than mycetocytes were detected.

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