Discovery of an alternate metabolic pathway for urea synthesis in adult *Aedes aegypti* mosquitoes

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We demonstrate the presence of an alternate metabolic pathway for urea synthesis in *Aedes aegypti* mosquitoes that converts uric acid to urea via an amphibian-like uricolytic pathway. For these studies, female mosquitoes were fed a sucrose solution containing $^{15}NH_4Cl$, [5-^{15}N]-glutamine, [^{15}N]-proline, allantoin, or allantoic acid. At 24 h after feeding, the feces were collected and analyzed in a mass spectrometer. Specific enzyme inhibitors confirmed that mosquitoes incorporate ^{15}N from $^{15}NH_4Cl$ into [5-^{15}N]-glutamine and use the ^{15}N of the amide group of glutamine to produce labeled uric acid. More importantly, we found that [$^{15}N_2$]-uric acid can be metabolized to [^{15}N]-urea and be excreted as nitrogenous waste through an uricolytic pathway. *Ae. aegypti* express all three genes in this pathway, namely, urate oxidase, allantoinase, and allantoicase. The functional relevance of these genes in mosquitoes was shown by feeding allantoin or allantoic acid, which significantly increased unlabeled urea levels in the feces. Moreover, knockdown of urate oxidase expression by RNA interference demonstrated that this pathway is active in females fed blood or $^{15}NH_4Cl$ based on a significant increase in uric acid levels in whole-body extracts and a reduction in [^{15}N]-urea excretion, respectively. These unexpected findings could lead to the development of metabolism-based strategies for mosquito control.

nitrogen metabolism | RNA interference

In vertebrates, as well as in insects, uric acid is produced by degradation of purines (1–4). A key enzyme in this pathway is xanthine dehydrogenase (XDH). It catalyzes the conversion of hypoxanthine to xanthine, which is then further degraded to uric acid (5). XDH activity has been studied in several insects including *Bombyx mori* (6), *Drosophila melanogaster* (7, 8), *Aldrichina grahami* (9), and *Aedes aegypti* (10, 11).

The use of [1-^{14}C]-glycine, [2-^{14}C]-glycine, and [^{14}C]-sodium formate permitted the determination of the origin of the five carbon atoms of uric acid in the bloodsucking insect *Rhodnius prolixus* and showed that the carbon atoms of uric acid in insects have the same origin as those reported in vertebrates (12).

Although uric acid can be excreted without any modification, it can also be metabolized into several nitrogen compounds. In some animals, uric acid can be converted to allantoin, allantoic acid, urea, and ammonia by reactions catalyzed by urate oxidase (UO), allantoinase (ALN), allantoicase (ALLC), and urease, respectively (13). (In this article the term "ammonia" refers to both NH_3 and NH_4^+ or a combination of the two.)

The final product of uric acid catabolism is unknown in insects, although UO (14) and ALN (15) activities have been reported, as has the excretion of allantoin and allantoic acid (16). The production of urea in insects has been attributed to arginase, which catalyzes the hydrolysis of arginine to form urea and ornithine. However, unlike in vertebrates, where arginine is generated in the urea cycle, the action of arginase in insects is limited to arginine from dietary sources or from endogenous protein turnover (3, 11, 17, 18). This is because insects lack one or more genes encoding enzymes required for the urea cycle. For example, mosquitoes lack the gene encoding ornithine carbamoyltransferase (19), which reacts with ornithine and carbamoyl phosphate to produce citrulline.

We previously reported that mosquitoes dispose of toxic ammonia through glutamine (Gln) and proline (Pro) synthesis, along with excretion of ammonia, uric acid, and urea (20). By using labeled isotopes and mass spectrometry techniques (21), we have recently determined how the ^{15}N from $^{15}NH_4Cl$ is incorporated into the amide side chain of Gln, and then into Pro, in *Ae. aegypti* (22). In the present article we demonstrate that the nitrogen of the amide group of Gln contributes to uric acid synthesis in mosquitoes and, surprisingly, that uric acid can be converted to urea by an amphibian-like uricolytic pathway.

Results

Incorporation of ^{15}N from $^{15}NH_4Cl$, [5-^{15}N]-Gln, and [^{15}N]-Pro into [^{15}N]-Urea. Twenty-four hours after feeding mosquitoes with 80 mM $^{15}NH_4Cl$, [5-^{15}N]-Gln, or [^{15}N]-Pro, unlabeled urea and urea labeled at one position were observed in the mosquito feces. The concentration of unlabeled urea after feeding with labeled isotopes did not change significantly compared with that observed after feeding with sucrose: 1.16 ± 0.17 nmol per animal (data not shown). Instead, urea labeled at one position reached levels of 0.50 ± 0.14 nmol per animal and 1.66 ± 0.35 nmol per animal after feeding with 80 mM $^{15}NH_4Cl$ and 80 mM [5-^{15}N]-Gln, respectively (Fig. 1). Similar effects were observed when mosquitoes were fed with 80 mM [^{15}N]-Pro, although the amount detected of urea labeled at one position was 0.85 ± 0.20 nmol per animal (Fig. 1). The quantification of unlabeled and labeled urea in mosquito feces was performed as indicated in *Materials and Methods* [see also supporting information (SI) Table 1]. In the feces, 13.95 ± 1.08 nmol of [5-^{15}N]-Gln per animal and 32.18 ± 2.69 nmol of [^{15}N]-Pro per animal was also detected at 24 h after feeding with 80 mM [5-^{15}N]-Gln and 80 mM [^{15}N]-Pro, respectively (data not shown), indicating that mosquitoes were not able to metabolize all of the [5-^{15}N]-Gln or [^{15}N]-Pro that was consumed.

Kinetics of Incorporation of ^{15}N from [^{15}N]-Pro into [5-^{15}N]-Gln. To verify whether labeled nitrogen from Pro can lead to labeled urea via [5-^{15}N]-Gln, we measured the incorporation of ^{15}N from [^{15}N]-Pro into [5-^{15}N]-Gln in the whole-body mosquito. Immediately after feeding mosquitoes with 30 mM [^{15}N]-Pro, the labeled proline from whole body decreased significantly through the time course and reached the lowest values at 24 h after

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The authors declare no conflict of interest.

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Data deposition: The nucleotide sequences reported in this paper for *Aedes aegypti* allantoicase, allantoinase, and urate oxidase genes have been deposited in the GenBank database (accession nos. EF676028, EF676029, and EF676030, respectively).

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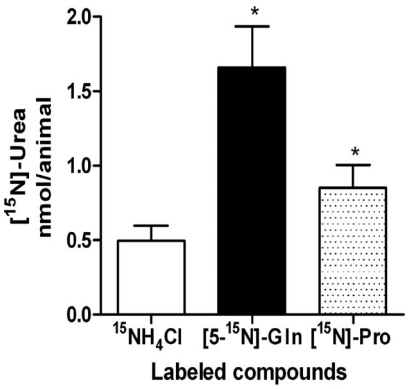

Fig. 1. Effect of 80 mM $^{15}NH_4Cl$, [5-^{15}N]-Gln, or [^{15}N]-Pro on urea synthesis in *Ae. aegypti* mosquitoes. [^{15}N]-urea concentrations were measured in the mosquito feces 24 h after feeding with 80 mM $^{15}NH_4Cl$, [5-^{15}N]-Gln, or [^{15}N]-Pro. Data are presented as mean ± SE of three independent samples. *, $P < 0.05$ when compared with $^{15}NH_4Cl$ by ANOVA.

feeding (Fig. 2*A*). Similar effects were also observed after feeding with 80 mM [^{15}N]-Pro. In contrast, the concentration of [5-^{15}N]-Gln increased from 0 to 6 h and then decreased at 24 h after feeding with either 30 mM or 80 mM [^{15}N]-Pro (Fig. 2*B*). The data indicate that mosquitoes can metabolize some of [^{15}N]-Pro into [5-^{15}N]-Gln.

Effect of Inhibitors on Urea Synthesis. We used methionine sulfoximine, an inhibitor of glutamine synthetase, and allopurinol, a specific inhibitor of XDH, to determine whether mosquitoes require the synthesis of [5-^{15}N]-Gln and labeled uric acid to produce urea labeled at one position. At 24 h after feeding mosquitoes with 80 mM $^{15}NH_4Cl$ in the presence of 20 mM methionine sulfoximine (Fig. 3*A*), or after feeding with 80 mM [5-^{15}N]-Gln and 1 mM allopurinol (Fig. 3*B*), the concentration of urea labeled at one position was significantly lower than it is in the absence of inhibitor, confirming that both glutamine and uric acid are involved in the urea synthesis.

Low-Energy Collision-Induced Dissociation Spectra of Uric Acid. Uric acid labeled at two positions was detected in the mosquito feces 24 h after feeding with 80 mM $^{15}NH_4Cl$ or [5-^{15}N]-Gln. Spectra of protonated unlabeled uric acid or labeled uric acid standards were compared with that of labeled uric acid observed in the feces after feeding with labeled isotopes. Among all of the fragments observed, there are two characteristic peaks that are indicative of labeled nitrogen at position 1 or position 3 (SI Table 2). In unlabeled uric acid standard ($m/z = 169$), the peaks at 152, 141, and 126 are due to neutral losses of NH_3 (17 Da), CO (28

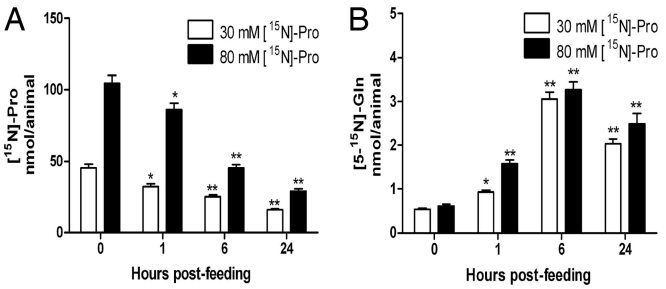

Fig. 2. Time course of whole-body labeled amino acids from *Ae. aegypti* after feeding with 30 or 80 mM [^{15}N]-Pro. (*A*) [^{15}N]-Pro. (*B*) [5-^{15}N]-Gln. Data are presented as mean ± SE of three independent samples. *, $P < 0.05$; **, $P < 0.01$ [when compared with 0 (immediately after feeding) by ANOVA].

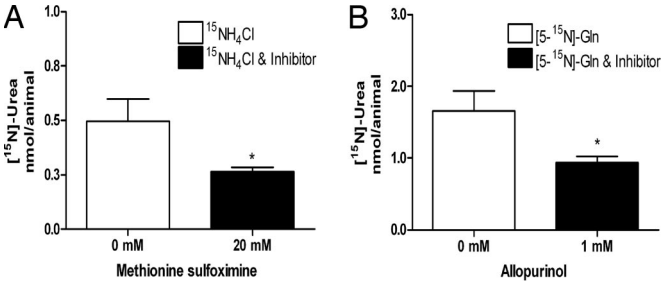

Fig. 3. Effect of specific inhibitors on [^{15}N]-urea synthesis. [^{15}N]-urea concentrations were measured in the mosquito feces 24 h after feeding with 80 mM $^{15}NH_4Cl$ and 20 mM methionine sulfoximine (*A*) or 80 mM [5-^{15}N]-Gln and 1 mM allopurinol (*B*). Control mosquitoes (0 mM) were fed in the absence of inhibitor. Data are presented as mean ± SE of three independent samples. *, $P < 0.05$ (when compared with 0 mM by Student's *t* test).

Da), and NHCO (43 Da), as was shown by Frycak *et al.* (23), whereas in [1, 3-$^{15}N_2$]-uric acid standard ($m/z = 171$), peaks at 153, 143, and 127 correspond to neutral losses of $^{15}NH_3$ (18 Da), CO (28 Da), and ^{15}NHCO (44 Da), respectively. According to the fragmentation mechanisms proposed for [2-^{13}C]-uric acid (24), it is possible to deduce that the neutral loss of ^{15}NHCO from [1, 3-$^{15}N_2$]-uric acid standard involves the ^{15}N at position 1. In the labeled uric acid obtained from the feces ($m/z = 171$), we detected two of the same peaks observed in labeled uric acid standards (153 and 143) but a peak of 128 instead of 127, which indicates losses of 18 Da ($^{15}NH_3$), 28 Da (CO), and 43 Da (NHCO). Therefore, it is possible to conclude that the neutral loss of NHCO in labeled uric acid from feces involves the N at position 1, whereas the neutral loss of $^{15}NH_3$ involves ^{15}N at position 3. The second labeled nitrogen in the uric acid from mosquito feces must be at position 7 or position 9. The lack of commercial labeled standards at those positions prevented us from knowing the exact location of the second labeled nitrogen in uric acid from feces; however, it should be located at position 9 as discussed below.

Effect of Allantoin and Allantoic Acid on Urea Synthesis. The effect of intermediate products of the uricolytic pathway on urea synthesis was analyzed by feeding mosquitoes with unlabeled intermediates. The concentration of unlabeled urea in the feces increased significantly and reached values of 2.48 ± 0.38 and 3.04 ± 0.33 nmol per animal after feeding with 10 and 30 mM allantoin, respectively (Fig. 4*A*). In the presence of allantoic acid, the unlabeled urea levels also increased significantly and reached values of 5.85 ± 0.56 and 13.31 ± 0.59 nmol per animal after

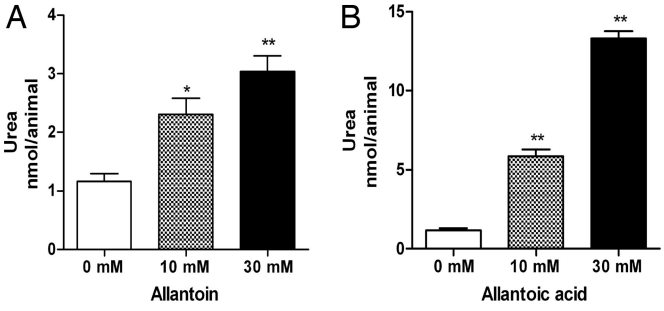

Fig. 4. Effect of allantoin and allantoic acid on urea synthesis. Urea concentrations were measured in the mosquito feces at 24 h after feeding. (*A*) After feeding with 0, 10, and 30 mM allantoin. (*B*) After feeding with 0, 10, and 30 mM allantoic acid. Control mosquitoes (0 mM) were fed only on 3% sucrose. Data are presented as mean ± SE of three independent samples. *, $P < 0.05$; **, $P < 0.01$ (when compared with 0 mM by ANOVA).

feeding with 10 and 30 mM allantoic acid, respectively (Fig. 4*B*). Taken together, the results indicate that mosquitoes are able to metabolize both allantoin and allantoic acid into urea.

The Gene Expression Patterns of UO, ALN, and ALLC in *Ae. aegypti* Mosquitoes (AaUO, AaALN, and AaALLC). To confirm that genes involved in the uricolytic pathway are present in mosquitoes, we searched *Ae. aegypti* genome and EST databases for homologous protein sequences using the BLAST program. A single-copy gene of each encoding AaUO, AaALN, and AaALLC was identified in the *Ae. aegypti* genome, and the corresponding full-length cDNA molecules were cloned and sequenced from the Rockefeller strain of *Ae. aegypti*. AaUO is composed of 325 residues. The deduced AaUO protein contains serine–lysine–leucine peroxisomal targeting residues in the C terminus. AaUO shares 85%, 58%, and 51% identity with *Anopheles gambiae*, *D. melanogaster*, and *B. mori*, respectively. The AaALN encodes a protein of 474 aa with 66%, 49%, and 52% sequence identity to *An. gambiae*, *D. melanogaster*, and *Ctenocephalides felis* homologs, respectively. The AaALN has all four histidine residues involved in zinc binding for catalytic activity, unlike *D. melanogaster* ALN (15). The AaALLC encodes a protein of 375 aa with 47% sequence identity to human ALLC. AaALLC homologs are also present in the insect genomes of *An. gambiae* and *B. mori*. However, *D. melanogaster* lacks the gene encoding ALLC.

The expression patterns of the AaUO, AaALN, and AaALLC genes in blood-fed *Ae. aegypti* mosquitoes were obtained with quantitative RT-PCR (QRT-PCR) experiments. As shown in Fig. 5, UO was constitutively expressed in the fat body (FB) throughout blood meal digestion, and the level increased ≈3-fold toward the end of digestion (Fig. 5*A*). In Malphigian tubules (MT), AaUO transcripts were absent before blood meal intake; however, they were induced in response to a blood meal, reaching the maximum level at 24 h after feeding and returning to the basal level by 72 h after feeding (Fig. 5*B*). The mRNA expression patterns of AaALN showed that the gene was down-regulated in FB and up-regulated in MT in response to a blood meal (Fig. 5 *C* and *D*). The mRNA expression patterns of AaALLC during the first gonotrophic cycle in *Ae. aegypti* were similar in both FB and MT (Fig. 5 *E* and *F*). The levels of AaALLC remained low during blood meal digestion and were dramatically increased in FB and MT at the end of digestion (Fig. 5 *E* and *F*). Induced expression of the UO, ALN, and ALLC genes in mosquitoes in response to blood meal feeding strongly argues for a functional role of the uricolytic pathway in urea synthesis. The expression patterns of the AaUO, AaALN, and AaALLC genes were also determined in response to feeding with 80 mM $^{15}NH_4Cl$. The highest transcript levels for AaUO were observed in FB and MT, respectively, at 1 and 6 h after feeding with $^{15}NH_4Cl$ (Fig. 5 *G* and *H*). The feeding of 80 mM $^{15}NH_4Cl$ alone had no effect on the mRNA level of AaALN and AaALLC in the FB or MT as compared with sucrose-fed mosquitoes (data not shown).

Concentration of Urea, Allantoin, and Allantoic Acid in the Feces of Blood-Fed Mosquitoes. It was previously reported that the ratio of nitrogen excreted as urea versus the nitrogen excreted as uric acid in mosquito feces at 72 h after blood meal feeding is ≈1:3 (11). The metabolic origin of this urea was thought to be exclusively from the arginase reaction. Based on our finding that *Ae. aegypti* can metabolize allantoin and allantoic acid to urea (Fig. 4) and that genes encoding the enzymes required for urea synthesis by the uricolytic pathway are expressed (Fig. 5), levels of urea, allantoin, and allantoic acid in the feces of blood-fed mosquitoes were measured. As shown in Fig. 6, blood-fed mosquitoes excrete allantoin and allantoic acid, but at lower concentrations than those at which they excrete urea. At 72 h after feeding, the levels of urea, allantoin, and allantoic acid

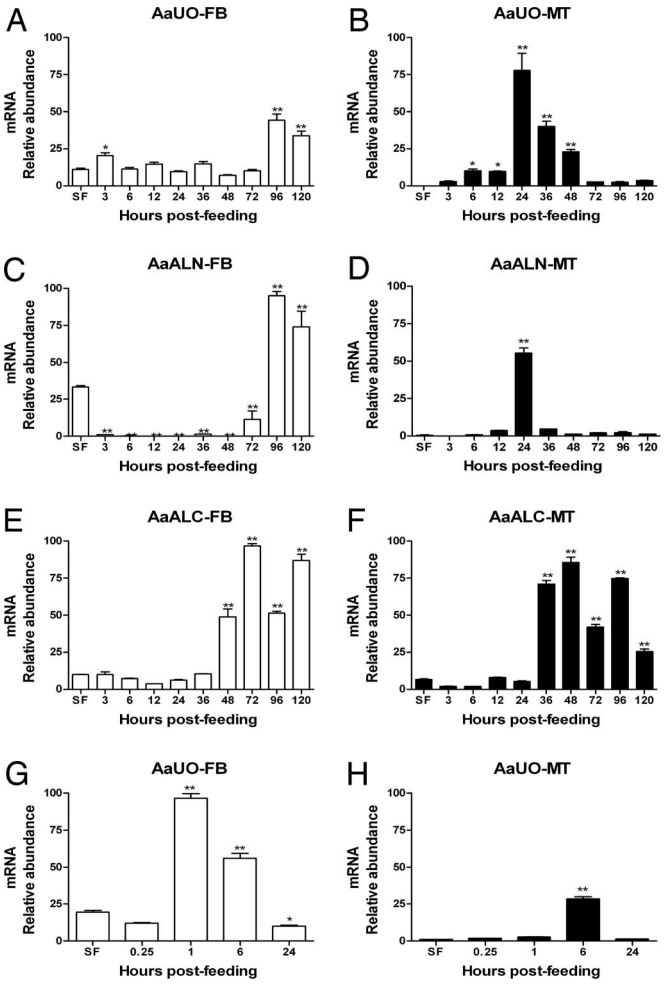

Fig. 5. The patterns of UO, ALN, and ALLC gene expression in *Ae. aegypti* females (AaUO, AaALN, and AaALLC). Shown is the relative abundance of mRNA in FB and MT tissues of mosquitoes after feeding with a blood meal (*A–F*) or after feeding with 80 mM $^{15}NH_4Cl$ (*G* and *H*). The mRNA levels were normalized according to the mRNA level of the S7 ribosomal protein. Sucrose-fed females (SF) were fed only on 3% sucrose. Data are presented as mean ± SE of three or five independent samples. *, $P < 0.05$; **, $P < 0.01$ (when compared with SF by ANOVA).

reached values of 130.67 ± 11.67, 16.39 ± 4.66, and 1.63 ± 0.23 nmol per animal, respectively. Taken together, these data support the physiological significance of the uricolytic pathway for nitrogen excretion in *Ae. aegypti* mosquitoes.

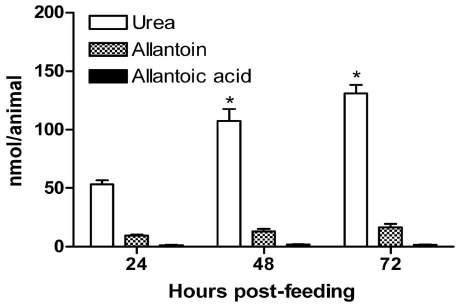

Fig. 6. Concentration of urea, allantoin, and allantoic acid in *Ae. aegypti* feces. Females were fed on blood meal, and the feces were analyzed at 24, 48, and 72 h after feeding. Data are presented as mean ± SE of five independent samples. *, $P < 0.05$ (when compared with 24 h by ANOVA).

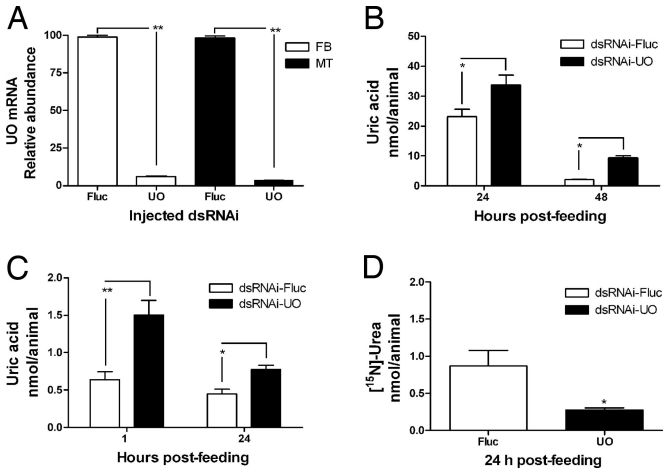

Fig. 7. Effect of UO knockdown on gene expression. *Ae. aegypti* mosquitoes were injected with dsRNAi-Fluc or dsRNAi-UO and then fed with a blood meal or 80 mM $^{15}NH_4Cl$. (*A*) Relative abundance of UO mRNA in tissues of injected mosquitoes at 24 h after feeding with a blood meal. The mRNA levels were normalized according to mRNA level of the S7 ribosomal protein. (*B*) Total uric acid concentration in whole-body mosquito at 24 and 48 h after feeding with a blood meal. (*C*) Total uric acid concentration in whole-body mosquito at 1 and 24 h after feeding with $^{15}NH_4Cl$. (*D*) [^{15}N]-urea concentrations in the mosquito feces at 24 h after feeding with $^{15}NH_4Cl$. Data are presented as mean ± SE of three independent samples. *, $P < 0.05$; **, $P < 0.01$ (when compared with dsRNAi-Fluc by Student's *t* test).

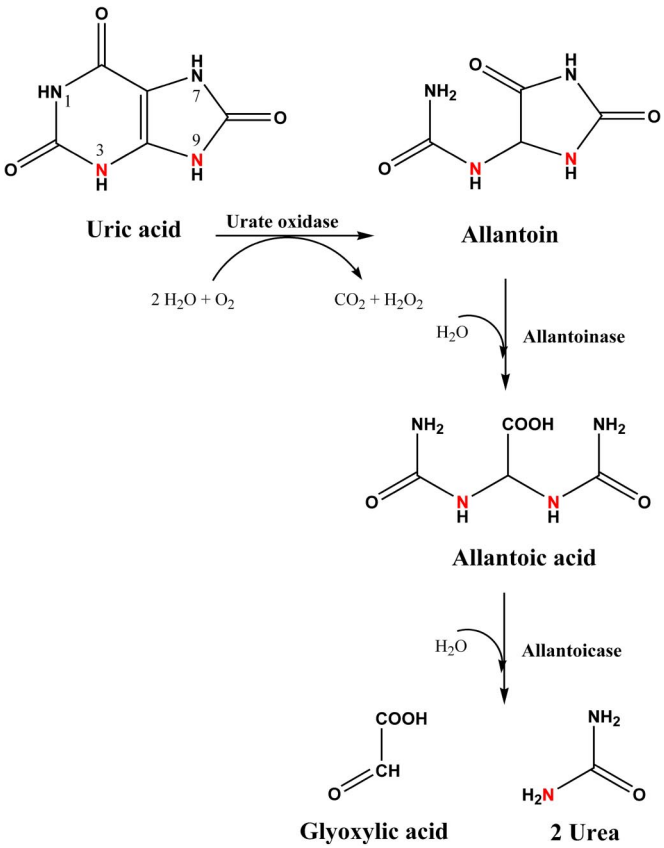

Fig. 8. Proposed metabolic pathway for uric acid degradation and urea synthesis in *Ae. aegypti* mosquitoes. The labeled nitrogen atoms are shown in red.

Effect of Knockdown of AaUO Gene Expression. To functionally validate the existence of a pathway leading to the synthesis of urea, we performed dsRNAi to knock down the AaUO gene in mosquitoes. At 24 h after feeding with a blood meal, the levels of AaUO mRNA were reduced by 94% in FB and 96% in MT of dsRNAi-UO injected mosquitoes compared with mosquitoes injected with the dsRNAi firefly luciferase (dsRNAi-Fluc) (Fig. 7*A*). Similar results were also obtained from injected mosquitoes that were fed with $^{15}NH_4Cl$ (data not shown). As shown in Fig. 7*B*, the total uric acid concentration in dsRNAi-UO-injected mosquitoes increased significantly at 24 and 48 h after blood meal compared with mosquitoes injected with the dsRNAi-Fluc control. When mosquitoes injected with dsRNAi-UO were fed with 80 mM $^{15}NH_4Cl$, the total levels of uric acid in whole body showed a pattern similar to that observed after blood meal (Fig. 7*C*). In addition, a significant decrease of labeled urea in feces was observed (Fig. 7*D*), confirming that mosquitoes require UO for urea synthesis.

Discussion

We have previously reported that feeding *Ae. aegypti* females with sucrose containing NH_4Cl results in ammonia detoxification by glutamine and proline synthesis, along with the excretion of ammonia, uric acid, and urea (20). In agreement with these results, we also showed that, by using $^{15}NH_4Cl$, the labeled nitrogen is first fixed into [5-^{15}N]-Gln by glutamine synthetase and assimilated by glutamate synthase to lead to [^{15}N]-Glu, which is converted to [^{15}N]-Pro in the mosquito (22). Moreover, we hypothesized that some of the [5-^{15}N]-Gln could be used for uric acid synthesis (22). We now show that in *Ae. aegypti* mosquitoes the nitrogen of the amide group of glutamine is involved in uric acid synthesis and that urea can be produced by uric acid catabolism.

Uric acid labeled at two positions was detected in the feces 24 h after feeding *Ae. aegypti* with $^{15}NH_4Cl$ or [5-^{15}N]-Gln. This finding is well correlated with the fact that [5-^{15}N]-Gln provides ^{15}N to uric acid at position 3 and also likely at position 9, as has been demonstrated in vertebrates (25, 26). In accordance with these results, we also observed that feeding mosquitoes with [^{15}N]-Pro led to the synthesis of [5-^{15}N]-Gln, which can be further used to synthesize labeled uric acid. These data confirm and extend the role of both glutamine and proline as a sink of nitrogen in mosquitoes (20, 22, 27, 28).

Our current studies also show that *Ae. aegypti* females are able to produce [^{15}N]-urea after feeding with $^{15}NH_4Cl$, [5-^{15}N]-Gln, or [^{15}N]-Pro, which shows that urea synthesis in mosquitoes is not limited to arginine cleavage by arginase. The data indicate that mosquitoes can use the ^{15}N from the amide group of two glutamine molecules to produce one molecule of uric acid labeled at two positions. This uric acid can be further metabolized to glyoxylic acid and two molecules of urea labeled at one position using a metabolic pathway catalyzed by UO, ALN, and ALLC (Fig. 8), as it occurs in amphibians and some fishes (13). The location of the two labeled nitrogen atoms at different rings in the uric acid molecule is consistent with the fact that when the labeled uric acid molecule is degraded it gives two urea molecules, each labeled at one position. In correlation with these observations, studies with inhibitors of glutamine synthetase or XDH showed a significant reduction of labeled urea concentration in feces. The results indicate that when glutamine synthetase is inhibited the levels of [5-^{15}N]-Gln decrease, which leads to a reduced production of labeled uric acid. Hence, less labeled uric acid is degraded, resulting in a reduction in the amount of labeled urea excreted. A decrease in the labeled urea levels was also observed when mosquitoes were fed with [5-^{15}N]-Gln and allopurinol, an inhibitor of XDH. These data demonstrate that both glutamine and uric acid are precursors in urea synthesis.

Although uric acid can be directly excreted in considerable amounts after a blood meal (10) as well as after feeding with

NH_4Cl (20), we have observed that some of the uric acid is retained in mosquito body and further metabolized to allantoin and allantoic acid, generating urea as a final product. Uric acid has been involved as a scavenger of free radicals in insects such as *D. melanogaster* (29), *R. prolixus* (30, 31), and *Pyrearinus termitilluminans* (32), but at present it is unknown whether uric acid has this function in mosquitoes.

The mRNA expression patterns of UO in FB and MT show that the gene expression is induced in both tissues after feeding blood or ammonia. More importantly, when the gene encoding UO was knocked down by dsRNAi, the levels of total uric acid in the whole body increased significantly, whereas the labeled urea concentration in the mosquito feces decreased significantly compared with the dsRNAi-Fluc control. These results confirm that mosquitoes have a functional UO that is required to degrade uric acid into urea.

Ramazzina *et al.* (33) have recently shown that in mammals uric acid can be oxidized by UO to 5-hydroxyisourate, which is converted to 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline to give allantoin. This can occur through two spontaneous reactions or by involving the two enzymes 5-hydroxyisourate hydrolase and 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase. Although *Ae. aegypti* mosquitoes have the genes encoding both enzymes (P.Y.S., J.I., and R.L.M., unpublished observations), it is unknown whether they are functionally significant and how they may affect the rate of urea synthesis.

The mRNA expression patterns of ALN and ALLC in *Ae. aegypti* mosquitoes show that they are expressed in FB and MT and regulated after blood meal. A significant increase in unlabeled urea excretion after feeding with allantoin and allantoic acid was observed that validates the functionality of the uricolytic pathway identified in mosquitoes.

Taken together, our data indicate that *Ae. aegypti* mosquitoes produce urea through two different metabolic pathways. One is through the action of arginase, which cleaves arginine to produce urea and ornithine (11). The other is through the uricolytic pathway shown in Fig. 8. Indeed, both pathways contribute to the urea pool. However, in the first case, the absence of ornithine carbamoyltransferase gene (19) means that the only source of arginine for urea production by arginase is from dietary sources or from endogenous protein turnover. In the second case, urea is derived from the degradation of uric acid, which is a depot for excess ammonia produced during the digestion of the blood. It is possible that relative flux through each pathway is tightly regulated. If so, then the coordinated operation of both pathways could be one of the essential processes that guarantees the survival of blood-fed mosquitoes.

Materials and Methods

Insects. *Ae. aegypti* (NIH-Rockefeller strain) were reared under standard conditions (27). Females were allowed to feed on 3% sucrose for the first 3–4 days and then starved for 24 h before experimental feeding. For nucleic acid microinjection, newly emerged *Ae. aegypti* female mosquitoes were used (34).

Sample Preparation for Mass Spectrometry Analysis. Females were placed individually in 10-ml plastic scintillation vials covered with nylon mesh and secured with rubber bands for feeding. Mosquitoes were allowed to feed on one of several solutions containing $^{15}NH_4Cl$, [5-^{15}N]-Gln, [^{15}N]-Pro (Isotec), allantoin, or allantoic acid (Sigma–Aldrich) in 3% sucrose for 15 min. Solutions were directly applied to the mesh in a volume of 5 μl. When DL-methionine, DL-sulfoximine, or allopurinol (Sigma–Aldrich) was added to the meal, the concentrations used are mentioned in the figure legends. Some mosquitoes were used to analyze the kinetics of incorporation of ^{15}N from [^{15}N]-Pro into [5-^{15}N]-Gln in the whole body, and the procedure used to prepare these samples was the same as described before (22). For feces analysis, 80 fed insects were kept in groups of 10 in plastic scintillation vials with access to water. At 24 h after feeding, the insects were removed from the vials. The feces of the 80 females were collected by washing the eight vials with a final volume of 1 ml of water. The suspension was mixed by using ultrasonic agitation, dried by using a vacuum centrifuge, and then dissolved in 100 μl of water. For the quantification of labeled amino acids in the feces, 50 μl of feces in solution was mixed with 25 μl of 1 mM deuterium-labeled amino acids (Cambridge Isotope Laboratories). Then the solution was dried and derivatized as described previously (21, 22). For the quantification of unlabeled and labeled urea, 50 μl of feces in solution was mixed with 25 μl of 1 mM [^{13}C-$^{15}N_2$]-urea and dried. When the mosquitoes were fed on cow blood meal (for 15 min), only insects fed ad libitum were kept in groups of five in a vial with access to water. At 24, 48, and 72 h after feeding, the feces of the five females were collected by washing the vial with 0.5 ml of water. The suspension was mixed by using ultrasonic agitation and centrifuged twice at 14,000 $\times$ *g* for 30 min, and the supernatant was filtered through a Microcon centrifugal filter device, YM-30 (Millipore). For the quantification of urea, allantoin, and allantoic acid, 5 μl of feces in solution was mixed with 5 μl of 1 mM [^{13}C-$^{15}N_2$]-urea and 5 μl of 1 mM uniformly labeled ^{15}N-Allantoin 50% (Icon Services) and dried.

Nanoelectrospray Ionization Tandem Mass Spectrometry. The analyses were carried out in a TSQ-700 triple stage quadrupole mass spectrometer (Finnigan MAT) equipped with a nanospray ion source operating in the positive mode. All of the dried samples were dissolved at 10–20 μM in a spray solution of H_2O:CH_3OH (1:1; vol/vol) containing 1% acetic acid. The solutions were then sprayed into the mass spectrometer at a rate of 3–5 μl/min. The needle voltage used was 1.2–1.6 kV, and the skimmer was at ground potential. The capillary temperature was maintained at 200°C. Collision-induced dissociation was performed with argon (3 mTorr) using a collision energy of 25 eV. The instrument was tuned to unit mass resolution, and the mass spectra were acquired in the profile mode. Spectra of protonated unlabeled and labeled urea standards were analyzed. According to the fragmentation spectra of unlabeled urea standard (m/z = 61) and labeled urea standards, [$^{15}N_2$]-urea (m/z = 63), and [^{13}C-$^{15}N_2$]-urea (m/z = 64), neutral losses of 17 Da (NH_3) or 18 Da ($^{15}NH_3$) occur by amide bond cleavage to produce fragments of 44, 45, or 46 Da, respectively. When [^{15}N]-urea (m/z = 62) from the mosquito feces is fragmented, neutral losses of 17 and 18 occur and produce fragments of 45 and 44 Da. Thus, the quantification of unlabeled and labeled urea in the mosquito feces was performed by multiple-reaction monitoring scans using [^{13}C-$^{15}N_2$]-urea as internal standard (SI Table 1), whereas the identification of labeled uric acid was performed as indicated above (see also SI Table 2). Based on the fragmentation patterns of protonated unlabeled and labeled allantoin and unlabeled allantoic acid standards (data not shown), the quantification of allantoin and allantoic acid in mosquito feces was performed at 15 eV by multiple-reaction monitoring scans (SI Table 3). All of the urea and uric acid standards were obtained from Cambridge Isotope Laboratories.

Molecular Cloning and Identification of Genes Involved in the Uricolytic Pathway. A putative ortholog of the genes involved in the uric acid degradation pathway including UO (EC 1.7.3.3), ALN (EC 3.5.2.5), and ALLC (EC 3.5.3.4) was identified from *Ae. aegypti* (NIH-Liverpool strain) through a database search. TBLASTN searches were performed against the TIGR *Ae. aegypti* Database (http://tigrblast.tigr.org/cmr-blast) and the GenBank database (*Ae. aegypti* whole-genome shotgun sequence database) using *D. melanogaster* UO (GenBank accession no. P16163) (35), *C. felis* ALN (GenBank accession no. AAN72834) (15), and *An. gambiae* ALLC (GenBank accession no. EAA09264).

RNA Isolation and QRT-PCR. The mRNA expression levels of endogenous *Ae. aegypti* UO, ALN, and ALLC genes were examined by QRT-PCR as described previously (20). Briefly, the FB and MT were dissected from sucrose-fed mosquitoes and cow blood-fed mosquitoes at 3, 6, 12, 24, 36, 48, 72, 96, and 120 h after feeding. Both tissues were also dissected from mosquitoes at 15 min, 1 h, 6 h, and 24 h after feeding with 80 mM $^{15}NH_4Cl$. Total RNA was extracted from each sample by using TRIzol reagent (Invitrogen), and 0.5 μg of total RNA was reverse-transcribed by using oligo-$(dT)_{20}$ primer and reverse transcriptase (Promega). The cDNA was then used as a template for QRT-PCR with gene-specific primers (SI Table 4). QRT-PCR amplifications were carried out by using the 7300 Real-Time PCR System in a 96-well microliter plate. Data were analyzed by using ABI Prism 7300 SDS Software (Version 1.2.2; Applied Biosystems).

Nucleic Acid Microinjection. AaUO and Fluc gene-specific primers flanked with T7 promoter sequence (SI Table 4) were used to amplify DNA from mosquito cDNA and pGL3 vector (Promega), respectively. The PCR product was purified by using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences). Purified products were used as a template for transcription. The dsRNAi were synthesized by using the MEGAscript T7 High Yield Transcription Kit (Ambion) by following the instruction manual. All dsRNAi preparations

were quantified by measuring absorbance at 260 nm, checked for integrity on an agarose gel, and stored at −80°C until used. Newly eclosed females were injected with dsRNAi (250 ng) and allowed to recover for 4 days before feeding with 80 mM $^{15}NH_4Cl$ or a cow blood meal. The specificity of the knockdown was examined by using QRT-PCR as described above. At different times after feeding (as indicated in figure legends), mosquitoes were immersed in liquid nitrogen. Whole bodies of five mosquitoes were homogenized in 100 μl of 0.1% of lithium carbonate. The suspension was boiled for 1 min, mixed by using ultrasonic agitation, and centrifuged twice at 14,000 $\times$ *g* for 10 min. Then the supernatant was filtered through a Microcon centrifugal filter device, YM-30, for 30 min and used to measure the total uric acid concentration with an uric acid reagent kit (Point Scientific). For the quantification of [^{15}N]-urea in the mosquito feces, the samples were prepared as described above.

Statistical Analysis. Student's test and one-way ANOVA followed by Dunnett's multiple-comparison test were used. A P value <0.05 was considered significant. All of the statistical analyses were carried out by using Prism 4.0 software (GraphPad).

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