Nitrogen

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Nitrogen Metabolism in Non-Nodulated and Nodulated Soybean Plants Related to Ureide Synthesis

Takuji Ohyama *, Kahori Matsumoto, Haruka Goto, Akihiro Saito and Kyoko Higuchi

Department of Agricultural Chemistry, Tokyo University of Agriculture, Tokyo 156-8502, Japan; a3saito@nodai.ac.jp (A.S.); khiguchi@nodai.ac.jp (K.H.)
* Correspondence: to206474@nodai.ac.jp

Abstract: Soybean plants can fix atmospheric N_2 in the root nodule, a symbiotic organ with rhizobia. The primary forms of N transported from N_2 fixation are ureides, allantoate, and allantoin, supplemented with asparagine. The nitrate absorbed in the roots is transported to the shoots in the forms of NO_3^- and asparagine with a little portion of ureides. The concentrations of N-metabolites were analyzed by capillary electrophoresis after supplying various concentrations of urea, precursors of ureides, and allopurinol, an inhibitor of xanthine dehydrogenase, to investigate the ureide synthesis pathway in the roots. When the non-nodulated soybean plants were treated with 0–5 mM of urea, the concentrations of asparagine and glutamine in the xylem sap and the roots increased remarkably. In addition, allantoate concentration increased with the urea concentrations becoming higher. Allopurinol inhibited the accumulation of allantoate but did not affect the asparagine and glutamine accumulation in roots, stems, leaves, and xylem sap, supporting that allantoate is synthesized by purine degradation in roots the same as in the nodules. When ureide precursors were supplied to the nodulated soybean plants, the concentrations of asparagine and glutamine in the xylem sap and roots increased, suggesting that the ureide precursors were absorbed and assimilated to amides in the roots.

Keywords: allantoate; allantoin; allopurinol; asparagine; glutamine; hypoxanthine; soybean; urate; urea; xanthine

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1. Introduction

Soybean plants can use both the N fixed in the root nodules and the N absorbed from the roots. Soybean roots absorb the N mainly in NO_3^- under aerobic soil conditions. However, soybean roots can absorb NH_4^+ and some organic compounds such as urea and glutamine [1–3]. It has been established that the principal forms of N transport from nodules and roots are different [4,5]. Ureides, allantoate (allantoic acid), and allantoin are the principal N-transport compounds from soybean nodules. Ishizuka (1970) [6] and Kushizaki et al. (1964) [7] found that nodulated soybean roots transport large amounts of ureides to the shoots compared with non-nodulated roots, and there are high accumulations of ureides in the stems and petioles of nodulated soybean compared with non-nodulating isoline through growth stages. Then, by isotope-tracer experiments using $^{15}N_2$, the ureides are synthesized rapidly in the nodules from fixed N_2, and these are the main compounds transported from the nodules to the shoot [4,8–12]. On the other hand, NO_3^- and asparagine (Asn) are major compounds transported from soybean roots supplied with nitrate in the medium [4,13–15]. However, 10–20% of the fixed N in the nodules is transported in Asn, whereas about 10% of the absorbed N in the roots is translocated in the form of ureides [4,16].

Non-nodulated soybean plants synthesize allantoin and allantoate, possibly via the de novo purine synthesis and degradation pathway [17,18]. Ureides synthesized in the nodules and roots are loaded in the xylem to the transpiring leaves [19,20]. The allantoin and

allantoate derived from the roots via the xylem are degraded into ammonium and assimilated into amino acids and protein in the leaves [17,18,21], and some of the N is re-transported to the pods and other sink organs, mainly in the form of Asn [14,17,18,22–25]. Ureides can be temporarily stored in the stem and petioles [26]. Alternatively, some of the ureides may be stored by incorporation into vegetative storage proteins in the leaves [27]. Atkins and Beevers (1990) suggested that ureides in the leaves are loaded into the leaf phloem to supply them to the developing sinks, including leaves, root tips, pods, or seeds [20], but Ohyama and Kawai (1983) reported that the ureides are not directly re-transported from leaves as it is [24]. Some ureides may be transferred from the xylem to the phloem for rapid supply to the sink organs [22]. Recently, ureide permeases have been identified in French beans [28] and soybean [29] that mediate the export of allantoin and allantoic acid from nodules to the xylem.

Ureides, allantoin, and allantoate are universal metabolites from purine degradation in animals, plants, and microorganisms. The ureides were detected first in the xylem sap of *Phaseolus vulgaris* and *Acer pseudoplatanus*, and these were found in the roots of the members of Boraginaceae, Plantanaceae, Hippocastanaceae, Aceraceae, and Leguminosae [12]. Ureides play important roles in the storage and translocation of N in maple (*Acer saccharum*) and comfrey (*Symphytum officinale*) [12,30,31]. In Acer, almost 100% of the N in the xylem sap was in allantoin and allantoate during the spring when N is required for new leaf growth [12].

Plants possess a complete oxidative purine catabolism pathway, with purine structures degraded via urate and allantoin to CO_2, glyoxylate, and NH_3 [32]. Figure 1 shows the purine degradation pathway in plants. All purine nucleotides metabolize to xanthine before purine ring cleavage. Two possible routes have been suggested for xanthine formation: one is via hypoxanthine, and another is through guanine. The latter route appears to be involved in the ureides biosynthesis in legume nodules [32,33].

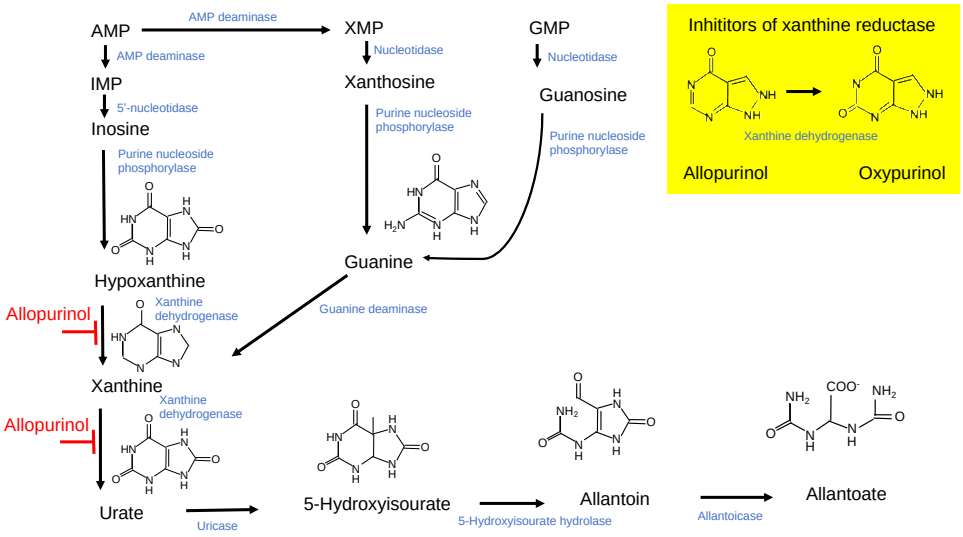

Figure 1. Purine degradation pathway of plants. Metabolites are shown in black letters, and enzyme names are shown in blue letters. The yellow box shows allopurinol, a competitive inhibitor of xanthine reductase, and the metabolite, oxypurinol produced by the reaction of xanthine dehydrogenase. Abbreviations: AMP: Adenosine monophosphate, XMP: Xanthosine monophosphate, GMP: Guanosine monophosphate, IMP: Inosine monophosphate.

Tracer experiments with ^{14}C-labeled precursors of ureides indicated that significant radioactivity was found in allantoin after being supplied the ^{14}C-glycine, but not by either ^{14}C-glyoxylic acids or ^{14}C-urea [12,34]. This result strongly supports the ureide synthetic pathways via purine synthesis and degradation [12]. Similar results were observed in soybean nodules [35]. These labeling studies supported that roots could synthesize ureides,

and most of the evidence favored the synthesis via purine degradation, although other pathways could not be discounted [12]. Mothes (1961) [36] and Reinbothe (1962) [30] have discussed another plausible allantoin synthesis, the direct formation of allantoate via condensation of urea and glyoxylate. This simple reaction from urea and glyoxylate to allantoate was supported by the finding that when [^{14}C] urea was fed to banana leaves, allantoate was heavily labeled [37], and Brunel (1951) [38] suggested an enzymic synthesis of allantoate from urea and glyoxylic acid in higher fungi.

Purine degradation is by xanthine breakdown pathways (Figure 1). Xanthine oxidoreductase (XOR) is a highly conserved family of molybdoflavoenzymes that are widely distributed from prokaryotic to eukaryotic organisms and considered to derive from a common ancestral progenitor [39]. In most living beings, the catabolism of hypoxanthine and xanthine to uric acid is catabolized by the xanthine dehydrogenase, but only mammals possess the xanthine oxidase [40]. Triplett et al. (1982) [41] purified and characterized soybean nodule xanthine dehydrogenase.

Allopurinol (4-hydroxypyrazolo(3,4-*d*)pyrimidine), a structural isomer of hypo xanthine, has been long used as a medicine for gout/hyperuricemia [42] to reduce urate concentrations in the blood through the inhibition of hypoxanthine to xanthine and xanthine to urate (red letters in Figure 1). Allopurinol competitively inhibits XOR and is converted into a single active metabolite, oxypurinol (yellow box in Figure 1). Oxypurinol has the same inhibitory effect as allopurinol but a much longer elimination half-life than the parent compound allopurinol [43,44].

Fujihara and Yamaguchi (1978) [45] investigated the effects of allopurinol application on the ureide metabolism of soybean plants. Allopurinol caused a significant drop in allantoin and allantoate in the stems and nodules of nodulated soybean plants, accompanied by the accumulation of xanthine in the nodules. Their results suggested that the main pathway of ureide formation in soybean nodules is purine degradation via xanthine to uric acid. However, the pathways of ureide synthesis in soybean roots are not fully elucidated; although non-nodulated soybean plants transport allantoate and allantoin in xylem sap, the levels were much lower than in nodulated soybean [46]. In this report we investigated the ureides synthesis pathway in the roots using absorbed N.

In our previous research, the effects of NO_3^-, NH_4^+, urea, or NO_3^- + NH_4^+ supply on the N-metabolite concentrations in xylem sap and each organ were compared between nodulated and non-nodulated soybeans. The concentrations of allantoate in the roots were the highest by urea treatment compared with others [46]. In this report, we investigated the effects of the urea concentrations on the N-metabolite concentrations in non-nodulated soybeans in Experiment 1. Then, the ureide synthesis pathway was evaluated in non-nodulated soybeans using allopurinol in Experiment 2. Finally, in Experiment 3, the effects of the application of ureide precursors and allopurinol on N-metabolites were investigated with nodulated soybean, in which a major site of ureide synthesis is the root nodules.

2. Materials and Methods

2.1. Plant Cultivation and Inoculation of Rhizobia

Soybean plants (*Glycine max* (L.) Merr.), cultivar "Williams", were inoculated or not inoculated with *Bradyrhizobium diazoefficiens* (strain USDA 110), and cultivated in a biophotochamber (LH-350S, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) at 28 °C day/18 °C night temperatures, 55% relative humidity, and under a photoperiod of 16 h light (228 μmol photons m^{-2} s^{-1})/8 h darkness.

For nodulated (Nod) soybean cultivation, the seeds were sterilized with 70% ethanol and 0.5% sodium hypochlorite solution, then washed with water. Then the seeds were inoculated with a suspension of *B. diazoefficiens* (10^8 cells mL^{-1}). Ten days after planting (DAP), the plant seedling was transplanted into 800 mL in a 900 mL glass bottle with continuous aeration. The glass bottle was covered with aluminum foil for shading the culture solution and roots. A newly developed nitrogen-free nutrient solution containing

$KHCO_3$ to stabilize the pH was used [47], and the culture solutions were renewed every 3 days.

The non-nodulated (non-nod) plants were cultivated in a separate chamber from the nodulated plants to prevent infection by rhizobia. The non-inoculated plants were cultivated in the same manner as Nod plants with the culture solution containing 1 mM $NaNO_3$ to support the N demand without nitrogen fixation until 27 DAP. Then the plants were cultivated with an N-free solution from 27 to 30 DAP just before the N treatments to lower the concentrations of NO_3^- and related N metabolites in the non-nodulated plants. First, the non-nod plants at 30 DAP were treated with different concentrations of urea in a culture solution (Experiment 1). Second, non-nodulated plants were treated with allopurinol, a potent inhibitor of xanthine oxidase, and urea was supplied (Experiment 2). Third, nodulated plants were treated with allopurinol and ureide precursors (Experiment 3).

2.2. Effect of Urea Concentrations on N Metabolite Concentrations in Non-Nodulated Plants

The urea treatments were as follows: Control, N-free solution (0 mM N urea); 0.5 mM urea (1 mM N urea), 1 mM urea (2 mM N urea), 2.5 mM urea (5 mM N urea), and 5 mM urea (10 mM N urea). After 24 h of the N treatments at 31 DAP, the basal part of the stem was cut by a razor blade, and the xylem sap was collected in glass wool in a plastic tube on the cut end of the main stem [2,3,48]. The xylem sap consists of xylem transport compounds from the roots to the shoots. The shoots and roots were dried using a freeze dryer (VD-400F, TAITEC, Saitama, Japan) and separated into leaves, stems plus petioles, and roots. Then, the dry sample was ground into a fine powder.

2.3. Effect of Allopurinol Treatments on N Metabolite Concentrations in Non-Nodulated Plants

The allopurinol treatments were as follows: Control, N-free solution without allopurinol; 0.1 mM allopurinol; 1 mM allopurinol; 5 mM urea without allopurinol; 0.1 mM allopurinol + 5 mM urea; and 1 mM allopurinol + 5 mM urea. After 24 h of the treatments at 31 DAP, the xylem sap was collected for 1 h, the same as in Section 2.2. The plant roots were washed and the shoots and roots of the plants were dried, separated into leaves, stems plus petioles, and roots, and ground into a fine powder.

2.4. Effect of Allopurinol and Ureide Precursors on N Metabolite Concentrations in Nodulated Plants

The Nod plants at 30 DAP were treated with ureide precursors or allopurinol for 24 h. The treatments were as follows: Control, N-free solution; 1 mM allantoin; 1 mM xanthine; 1 mM hypoxanthine; 1 mM urate; and 1 mM hypoxanthine + 1 mM allopurinol. After 24 h of the treatments at 31 DAP, the xylem sap was collected for 1 h, the same as in Section 2.2. The plant roots were washed and the shoots and roots of the plants were dried, separated into leaves, stems plus petioles, roots, and nodules, and ground into a fine powder.

2.5. Analysis of the Principal N Metabolites

Approximately 25 mg of sample powder was extracted with 1 mL of 80% ethanol containing 0.2 mM MES (2-(N-morpholino)ethanesulfonic acid) as an internal standard for the analysis by the capillary electrophoresis [45,49]. Then, the ethanol extract was evaporated in a vacuum and redissolved in 1 mL of pure water, then filtered by a 0.45 μm membrane filter before injection.

The concentrations of nitrate, glutamate (Glu), aspartate (Asp), glutamine (Gln), asparagine (Asn), allantoin, and allantoate in the xylem sap and extracts were analyzed by capillary electrophoresis (7100, Agilent Technologies, Inc., Santa Clara, CA, USA). A fused silica tube (inner diameter (id): 50 μm; length: 104 cm) and a commercial buffer solution (α-AFQ109, Ohtsuka Electronics Co., Ltd., Osaka, Japan) were used with an applied voltage of −25 kV. Signal peaks were detected with a signal wavelength of 400 nm and a reference wavelength of 265 nm. The pherogram of the standard solution is shown in Figure S1. Hy-

poxanthine and allopurinol were analyzed by the same procedures, showing the negative peaks by this experimental condition.

The concentration of ammonium in the xylem sap and the extract was determined by the indophenol method using a microplate reader (SH-1000, Corona Electric, Co., Ltd., Ibaraki, Japan) [48,50]. The concentration of urea was measured by urease method, and it was calculated by subtracting the ammonium concentration after the urease reaction (ammonium + urea) from the ammonium concentration without the urease reaction [49].

2.6. Statistics

The experiments 1 and 2 were conducted with three biological replications, and experiment 3 was performed with four replications. The plants were randomly arranged in a growth chamber. Tukey's test was used for evaluating the statistical significance among the treatments. The statistical significance was determined using the statistical analysis program of Osaka University [51].

3. Results

3.1. Effect of Urea Concentrations on N-Metabolite Concentrations in Non-Nodulated Soybean Plants (Experiment 1)

In the first experiment, the non-nodulated soybean plants were supplied with various concentrations of urea on the N-metabolite concentrations including allantoin and allantoate. The non-nodulated soybean plants were cultivated with a solution containing 1 mM $NaNO_3$ until 27 DAP to support the N requirement without N_2 fixation. After the plants had been N-starved for 3 days, the 30 DAP plants were treated with 0, 0.5, 1.0, 2.5, and 5 mM of urea for 24 h. The control plants with 0 mM of urea depleted N for 4 days before sampling time, and the xylem sap exudation rate was low at 0.2 mL/h compared with other treatments around 0.6–0.7 mL/h.

Figure 2A shows the concentrations of the principal N-metabolites in the xylem sap of the non-nodulated soybean plants treated with various concentrations of urea. In the control plants with 0 mM of urea, almost all metabolites could not be detected, suggesting that 4 days of N starvation depleted the storage of N in the roots. When 0.5 mM urea was applied, the concentration of Asn was the highest at 39 mg N/L, followed by allantoate (16 mg N/L), and Asp (8 mg N/L). In the 1.0 mM urea treatment, the concentration of Asn was the highest at 119 mg N/L, followed by Gln (40 mg N/L), allantoate (24 mg N/L), and Asp (14 mg N/L). The concentrations of Asn were similar between 1 mM and 2.5 mM urea treatments but slightly decreased by the 5 mM urea treatment. Instead, the Gln concentrations became significantly higher in the 2.5 mM urea (127 mg N/L) and 5.0 mM urea (108 mg N/L) treatments. It was noteworthy that allantoin and Glu could not be detected in any urea treatments. The concentrations of urea and ammonium in xylem sap were highest in the 5 mM urea treatment.

In the control roots treated with 0 mM urea, the concentrations of principal N-metabolites were high in Asp (11 μg N/gDW), Asn (11 μg N/gDW), Allantoate (9 μg N/gDW), Glu (6.5 μg N/gDW), and Allantoin (2.6 μgN/gDW) (Figure 2B). The concentration of Asn increased with the concentrations of urea in the solution up to 1 mM, but it reached a plateau of around 400–500 μg N/gDW from 1 mM to 5 mM of urea. On the other hand, Gln was not detected in the 0 mM and 0.5 mM urea treatments, but the Gln concentrations increased consistently up to 5 mM urea, showing the maximum at 790 μg N/gDW with 5 mM urea. The concentration of allantoate increased up to 2.5 mM urea (55 μg N/gDW). The concentrations of Asp, Glu, urea, and ammonium increased along with the urea concentration in the medium, although the concentration of allantoin did not respond to the urea concentrations in the culture solution.

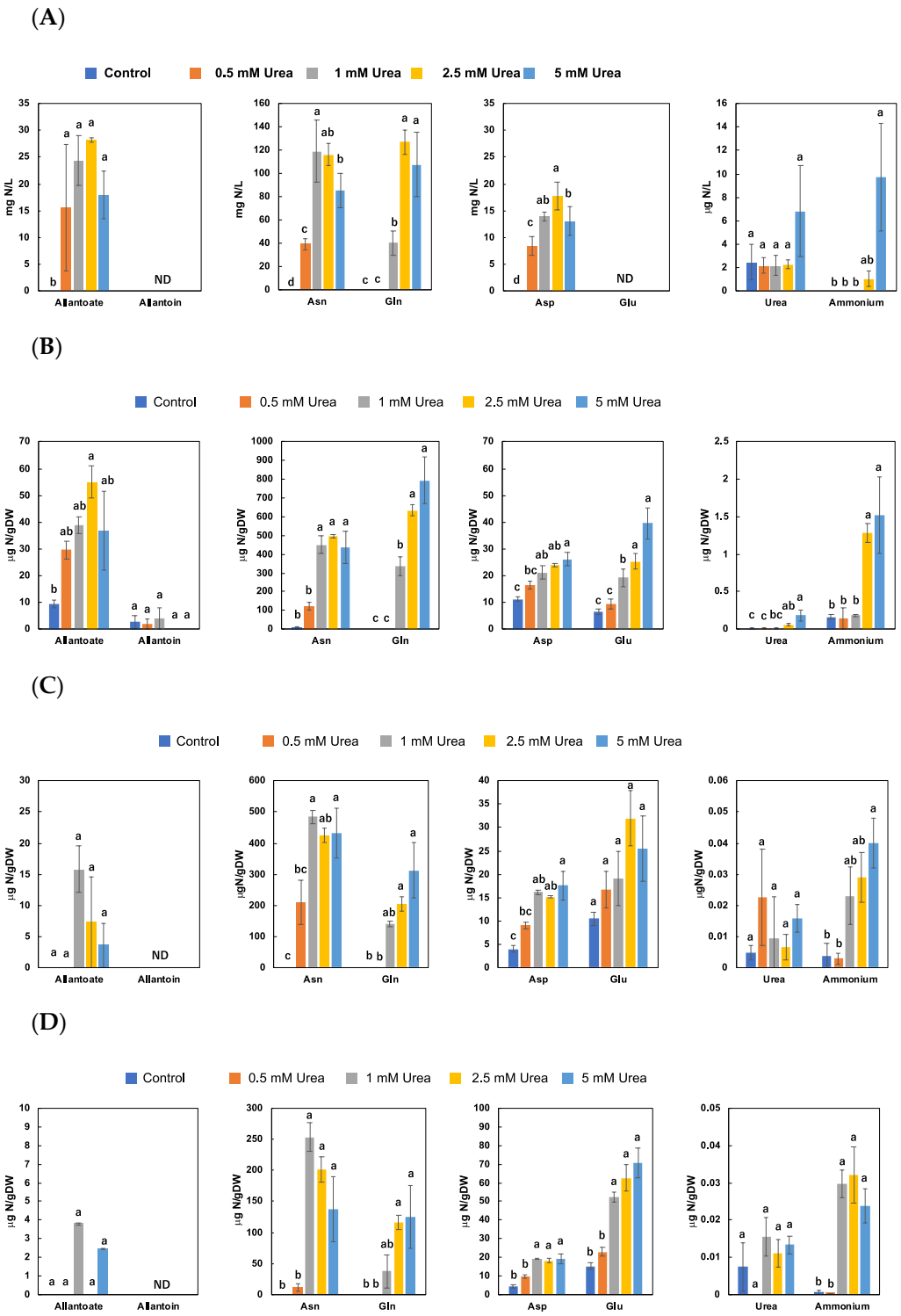

Figure 2. Comparison of the concentrations of the principal N-metabolites in the xylem sap of non-nodulated soybean plants treated with various concentrations of urea. (**A**) xylem sap, (**B**) roots, (**C**) stems, and (**D**) leaves. Treatments: control, control with N-free solution; 0.5 mM, 0.5 mM urea; 1 mM, 1 mM urea; 2.5 mM, 2.5 mM urea; and 5 mM urea. $N = 3$. Average $\pm$ standard error. The different letters on the top of the columns indicate significant differences in the N concentration among the treatments based on Tukey's test ($p < 0.05$). ND: not detected. Abbreviations; Asn: Asparagine, Gln: Glutamine, Asp; Aspartate, and Glu; Glutamate.

The patterns of the concentration changes of Asn, Gln, Asp, and Glu in the stems (Figure 2C) and in the leaves (Figure 2D) were similar to those in the roots (Figure 2B). Asn and Gln were two major N-metabolites. The concentration of Asn increased at 200 μg N/gDW with 0.5 mM urea and increased to 500 μg N/gDW with 1.0 mM urea, but it became constant in the stems or decreased in the leaves after the urea concentration was higher, 1 mM to 5 mM. The concentrations of Asp, Glu, and Gln increased with increasing the urea concentrations in the stems and leaves. Allantoate could be detected in the stems and leaves, with 1.0, 2.5, and 5.0 mM urea treatments, but allantoin was not detected in the stems and leaves, same as the xylem sap (Figure 2A).

In Experiment 1, the allantoate concentration in the xylem sap and the roots increased by elevating urea concentrations from 0 to 5 mM. Therefore, we use 5 mM urea for the next, Experiment 2, as an N source.

3.2. Effect of Allopurinol Treatments on N Metabolite Concentrations in Non-Nodulated Soybean Plants (Experiment 2)

The effects of 0.1 mM or 1 mM allopurinol treatment with the N-free (control) or 5 mM urea in the culture solution on the concentrations of the principal N-metabolites were investigated. Figure 3A shows the concentrations of N-metabolites in the xylem sap. Irrespective of allopurinol treatment, the principal N-metabolites were not detected in the xylem sap of the non-nod soybean, same as the control treatment of Experiment 1 (Figure 2A). When 5 mM urea was supplied with 0 mM allopurinol, the concentrations of Gln (123 mg N/L) and Asn (99 mg N/L) were high, followed by allantoate (34 mg N/L). Both allantoin and Glu were not detected in any treatments. Neither 0.1 mM nor 1 mM allopurinol treatment affected the concentrations of Asn and Asp, but the allopurinol treatments increased the Gln concentration. The concentration of allantoate was significantly decreased by the 0.1 mM allopurinol treatment, at (6 mg N/L) compared with the control (34 mg N/L), and allantoate was not detected with the 1 mM allopurinol treatment.

The effects of the 0.1 mM and 1 mM allopurinol treatments with or without 5 mM urea on the concentrations of major N-metabolites in the roots are shown in Figure 3B. Different from the xylem sap, about 10 mg N/gDW of Asp and Glu were detected in the roots with N-free conditions. When 5 mM urea was supplied, the concentrations of Gln and Asn were high, followed by allantoate. The allopurinol treatments with 5 mM urea were not affected by the Gln, Asn, Glu, and Asp concentrations, but allantoate was not detected in the roots with both the 0.1 mM and 1 mM allopurinol treatments.

Similar trends were observed in the stems (Figure 3C) and the leaves (Figure 3D). Treatment with 5 mM urea increased the concentrations of Asn, Gln, Asp, and Glu, but 0.1 mM and 1 mM allopurinol treatments did not affect them. On the other hand, allopurinol treatment depressed the allantoate accumulation in the stems and leaves.

In Experiment 2, allantoate was detected only when urea was supplied in the culture solution, and allantoate accumulation was depressed by the addition of allopurinol, supporting the idea that allantoate synthesis in the soybean roots is via purine degradation.

3.3. Effect of Allopurinol and Ureide Precursors on N Metabolite Concentrations in Nodulated Soybean Plants (Experiment 3)

In the third experiment, nodulated soybean plants were treated with precursors of ureides or allopurinol. In this case, allantoate (120 mg N/L) and allantoin (70 mg N/L) were the major N-metabolites in the xylem sap, followed by Asn (28 mg N/L) and Gln (18 mg N/L) in the control treatment with N-free solution (Figure 4A). When allanotin, xanthine, hypoxanthine, and urate (precursors of allantate) were supplied for 24 h, the concentrations of allanoin and allantoate were not significantly changed, but the concentrations of Gln, Asn, Asp, and Glu were significantly increased by these compounds. The Asn and Gln concentrations increased up to 100–200 mg N/L by supplying the ureide precursors, and several times higher than those in the control treatment. The levels of Asn and Gln were almost the same as that with the 2.5–5 mM urea supply (Figure 2). This suggests that allantoin, xanthine, hypoxanthine, and urate were absorbed from the soybean roots and

metabolized to amides and amino acids. The application of allopurinol strongly inhibited the accumulation of almost all of the N-metabolites in the xylem sap: allantate, allantain, Asn, Gln, Glu, but not Asp.

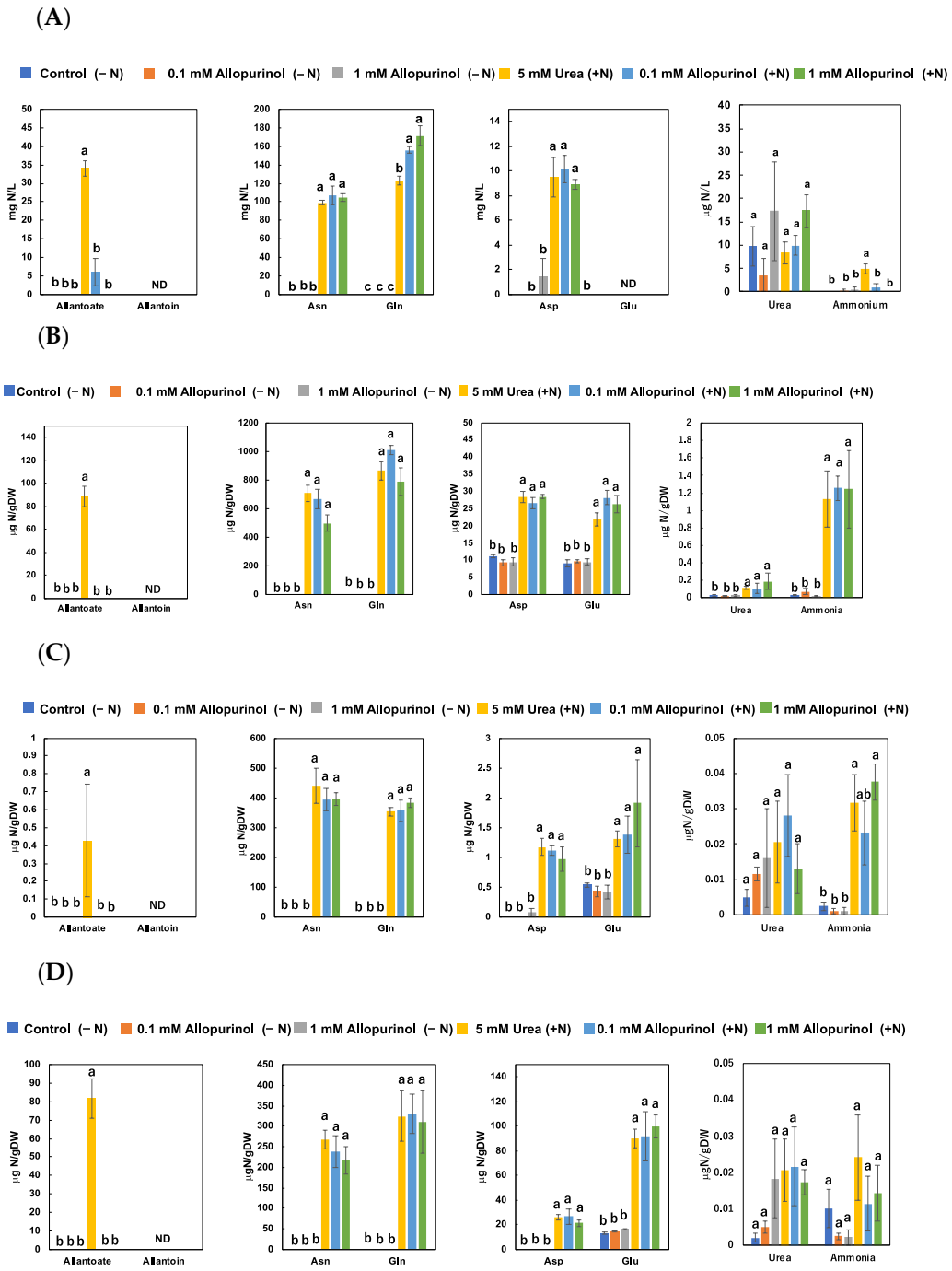

Figure 3. Concentrations of the principal N metabolites in the xylem sap of non-nodulated soybean plants treated with control without allopurinol, 0.1 mM allopurinol, 1 mM allopurinol (−N) with (+N) or without (−N) 5 mM urea. (**A**) xylem sap, (**B**) roots, (**C**) stems, and (**D**) leaves. $N = 3$. Average ± standard error. The different letters on the top of the columns indicate significant differences in the N concentration among the treatments based on Tukey's test ($p < 0.05$). ND: not detected. Abbreviations; Asn: Asparagine, Gln: Glutamine, Asp; Aspartate, and Glu; Glutamate.

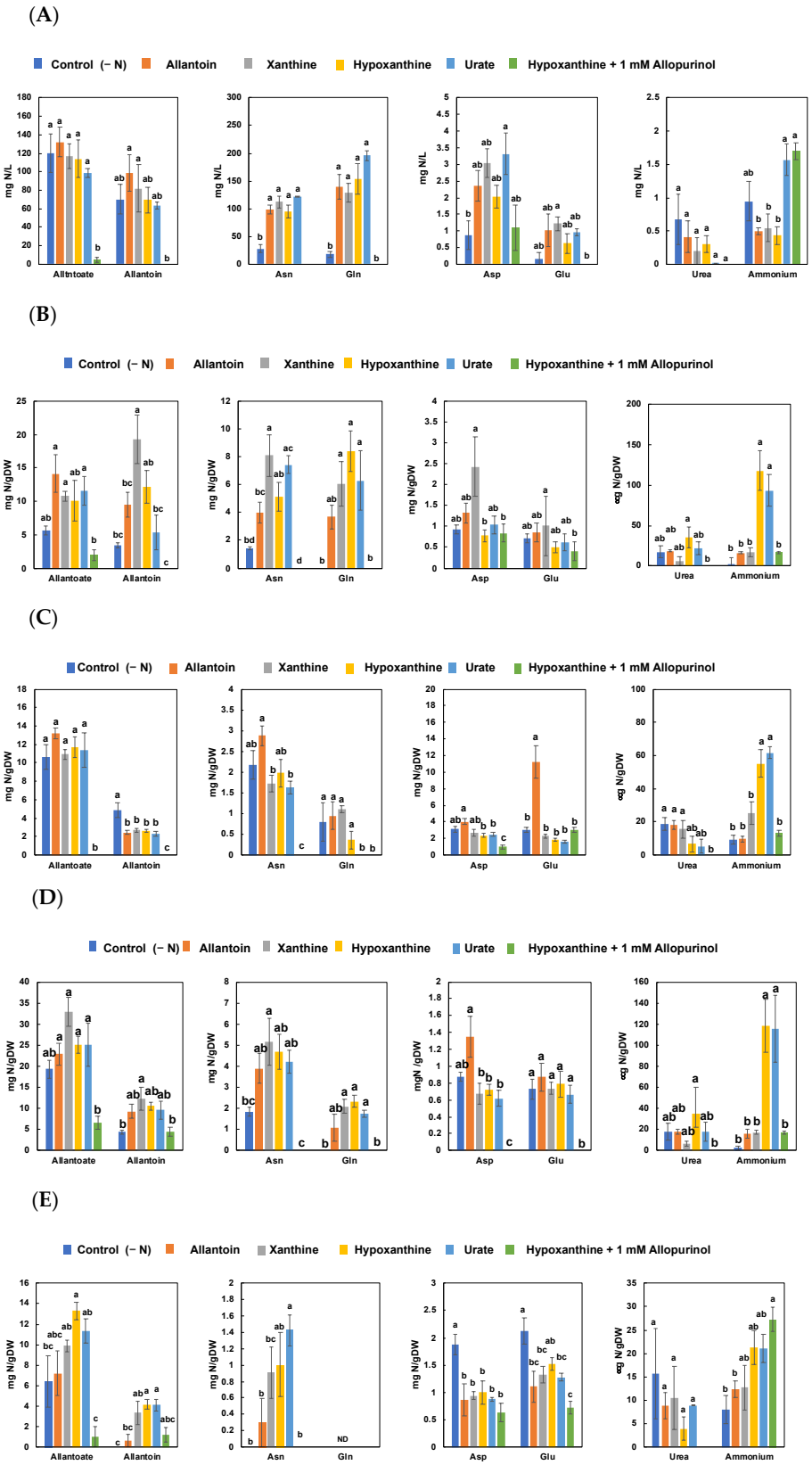

Figure 4. Concentrations of the principal N metabolites in the xylem sap of nodulated soybean plants treated with 1 mM allantoin, 1 mM xanthine, 1 mM hypoxanthine, 1 mM urate, or 1 mM hypoxanthine + 1 mM allopurinol with or without 5 mM urea. (**A**) xylem sap, (**B**) roots, (**C**) nodules, (**D**) stems, and (**E**) leaves. Average ± standard error. The different letters on the top of the columns indicate significant differences among the treatments based on Tukey's test ($p < 0.05$). ND: not detected. $N = 4$.

The concentrations of allantoate and allantoin were increased by precursor treatments in the roots of nodulated plants supplied with ureide precursors (Figure 4B). Furthermore, the concentrations of Gln and Asn increased with allantoate precursor treatments, but those of Asp and Glu were not. Allopurinol treatment strongly decreased the concentrations of ureides and amides, but not for amino acids.

The concentrations of allantoate, allantoin, Asn, Gln, Asp, and Glu in nodules were not significantly different after allantoate precursor treatments (Figure 4C). However, allopurinol almost completely inhibited the accumulations of allantoate, allantoin, Asn, and Gln in nodules, but not Glu.

The effects of the allantoate precursors on the concentrations of major N-metabolites in the stems (Figure 4D) and in the leaves (Figure 4E) are relatively the same. After a 24 h treatment of allantoate precursors, allantoate and allantoin concentrations were increased compared with control with the N-free solution. It was true for Asn and Gln, but not Asp and Glu like those in the roots (Figure 4B). Alloprinol treatment decreased the concentrations of allantate, allantoin, Asn, Gln, Asp, and Glu in the stems and leaves.

Figure 5 shows the concentrations of allopurinol and hypoxanthine in each part of soybean and xylem sap. Allopurinol was detected in the roots and leaves but not in the nodules and stems. Allopurinol was detected in the xylem sap, so the absorbed allopurinol in the roots was transported to the leaves through the xylem vessels. Hypoxanthine was also detected in the roots, nodules, leaves, and xylem sap only in Hypoxanthine + allopurinol treatment and not in the other treatments including Hypoxanthine treatment. This suggests that allopurinol inhibits hypoxanthine oxidation, and hypoxanthine was accumulated. Although allopurinol could not be detected in the nodules, the concentrations of allantate and allantoin in the xylem sap and nodules significantly decreased, and the accumulation of hypoxanthine occurred. These results indicate that a low concentration of allopurinol might inhibit the xanthine dehydrogenase in nodules, or oxypurinol derived from allopurinol might inhibit the XDH activity.

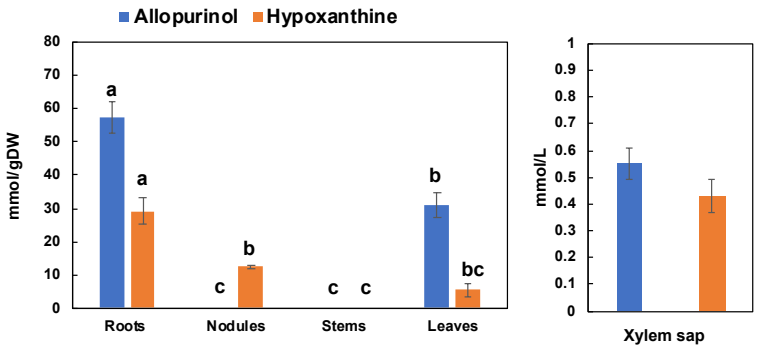

Figure 5. Concentrations of allopurinol and hypoxanthine in the roots, nodules, stems, leaves, and xylem sap of nodulated soybean plants treated with 1 mM hypoxanthine + 1 mM allopurinol. Average ± standard error. The different letters on the top of the columns indicate significant differences among organs based on Tukey's test ($p < 0.05$). $N = 4$.

In Experiment 3, allopurinol treatment strongly depressed allantoate transport in the xylem sap and accumulation in the nodules and other organs, suggesting that allopurinol might have been transported to the nodules and inhibited XDH and allantoate synthesis.

4. Discussion

4.1. Effect of Urea Concentrations on N-Metabolite Concentrations in Non-Nodulated Soybean Plants (Experiment 1)

When urea was supplied in the culture solution, urea itself was not transported via the xylem sap, but the amides, Asn, and Gln were the main transported forms (Figure 2A). The marked increase in Asn and Gln concentrations was observed in both nodulated and non-nodulated soybean plants supplied with 5 mM ammonium, 5 mM urea, and 2.5 mM

ammonium +2.5 mM nitrate supplied for 24 h but not with 5 mM nitrate [46]. The absorbed urea might be assimilated after being catabolized to ammonium and carbon dioxide by the enzyme urease in the roots, because the increase in urea concentration could not be observed in the xylem sap (Figure 2A). The ammonium is primarily assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway in the roots [46,52]. The finding was that when the low concentrations of urea (0.5 mM or 1 mM) were supplied, the Asn concentration in the xylem sap was higher than the Gln. However, the supply of higher concentrations of urea at 2.5 and 5 mM exhibited higher Gln concentrations relative to Asn, which was interesting, but the mechanisms are unknown.

The increase in the concentration of allantoate in the xylem sap (Figure 2A) and in the roots (Figure 2B) of the non-nodulated soybean by supplying urea supports the allantoate formation and transport from roots after urea was metabolized. The reason why allantoin has not been detected in the xylem sap and most organs except the roots is unknown, but it is possibly due to the rapid turnover rate from allantoin to allantoate. Cheng et al. (1999) [53] observed that alfalfa plants, which belong to an amide transporting group, metabolized ammonium to ureides in the roots. Among organs, the ureide concentration was higher in the lateral roots and in the nodules than in other tissues. In the main root, the ureide concentration increased gradually from the base to the root tip. This distribution trend of ureides is similar to that of the non-nodulating soybean variety [54].

4.2. Effect of Allopurinol Treatments on N Metabolite Concentrations in Non-Nodulated Plants (Experiment 2)

The second experiment considered the effect of the 0.1 mM and 1 mM allopurinol treatments on the metabolite concentrations in the non-nodulated soybean plants. When N was not supplied in the control (-N) treatment, almost no N-metabolites were detected in the xylem sap and every organ, with or without allopurinol. When 5 mM urea was applied, the control plants accumulated an appreciable amount of allantoate in each part and in the xylem sap. However, the 1 mM allopurinol treatments completely inhibited allantoate accumulation in the xylem sap (Figure 3A), and a small amount of allantoate was detected in the xylem sap with 0.1 mM allopurinol treatment. On the other hand, 1 mM and 0.1 mM allopurinol treatments did not repress the concentrations of Asn, Gln, Asp, and Glu in the xylem sap. This was true for the roots (Figure 3B), stems (Figure 3C), and leaves (Figure 3D). These results support the idea that the purine degradation pathway is the main route of allantoate synthesis in soybean roots.

4.3. Effect of Allopurinol and Ureide Precursors on N-Metabolite Concentrations in Nodulated Plants (Experiment 3)

In the third experiment, the precursors of ureides and allopurinol were administered to the nodulated soybean plants in which ureide synthesis mainly occurs in the root nodules. When 1 mM allantoin, xanthine, hypoxanthine, or urate was supplied to the culture solution, the concentrations of Asn and Gln were significantly increased in the xylem sap (Figure 4A) or the roots, stems, and leaves, suggesting that some of these compounds were absorbed in the roots and assimilated to Asn and Gln as well as the urea treatment in Experiment 1.

The hypoxanthine + allopurinol treatment strongly inhibited the flow of allantate and allantoin in the xylem sap. Furthermore, allantoate and allantoin were not detected in the nodules (Figure 4B), suggesting that allopurinol might be transported to the nodules and then inhibits the xanthine reductase for ureide synthesis in the nodules. Allopurinol was detected in the roots, leaves, and xylem sap (Figure 5). Although allopurinol was not detected in the nodules and stems, it inhibited the ureide synthesis in the nodules judged from the inhibitory effects on ureide synthesis. Appreciable hypoxanthine concentrations were detected with hypoxanthine + allopurinol treatment but not other treatments, suggesting that some xanthine might be accumulated by inhibition of allopurinol on xanthine reductase (Figure 1). However, Fujihara and Yamaguchi (1978) reported that while the allantoin and allantoate levels in nodules are high, the xanthine and uracil concentrations are negligible [45]. Collier and Tegeder (2012) [29] reported that ureide transporters control

the allantoin and allantoic acid levels in the nodules and that these ureides or related N compounds provide regulatory signals for the N_2 fixation, nodule metabolism, growth, and rhizobia infection.

Further, recent studies support that ureides may act as signals controlling plant responses to environmental stresses, including the regulation and coordination of primary metabolism and plant growth [25,55,56]. Lee et al. (2018) reported that the gene expressions of two genes involved in ureide metabolism, allantoinase and ureide permease 1, responded to the low N status in rice plants [55]. Redillas et al. (2019) further reported that allantoin accumulation through overexpression of ureide permease 1 improved rice growth under N-limited conditions [56]. While the mechanism behind these phenomena is unclear, Takagi et al. (2018) demonstrated that ureide degradation plays an important role in supporting healthy growth and development in non-legume Arabidopsis during and after the transition from vegetative to reproductive stages [57].

In the future, further research is required for the physiological roles of ureides in plants in addition to N metabolism and transport.

5. Conclusions

Metabolic pathways of allantoate in non-nodulated soybean roots were evaluated by supplying urea plus allopurinol, a potent inhibitor of xanthine reductase. When the non-nodulated soybean plants were treated with 0–5 mM urea in Experiment 1, the allantoate concentration in the roots and xylem sap increased with the elevated urea concentrations, so 5 mM urea was used as an N source for Experiments 2 and 3. Allopurinol strongly inhibited the allantoate accumulation in the roots of non-nodulated soybean plants but did not affect the Asn and Gln accumulation together with urea supply, supporting that allantoate is synthesized via the purine degradation pathway in the roots that were supplied with urea. When the nodulated soybean plants were treated with allopurinol, allantoate levels became significantly lower in the xylem sap and each organ, suggesting that allopurinol and/or oxypurinol might be transported to the nodules and inhibit the xanthine reductase in the nodules. The ureide synthesis pathways were similar in the roots and nodules, but further research is necessary to uncover the mechanism of why the N fixed in the nodules is mainly assimilated to the ureides while the N absorbed in the roots is assimilated to the Asn.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nitrogen4020014/s1, Figure S1. Pherogram of standard compounds by capillary electrophoresis. Standard solution composed of nitrate, as-partate, glutamate, allantoate, allantoin, asparagine, glutamine, hypoxanthine, allopurinol (100 mg/L), and MES (1 mM) was analyzed. 1; NO_3^-, 2; Asp, 3; Glu, 4; Allantoate, 5; MES, 6; Allantoin, 7; Hypoxanthine, 8; Asn, 9; Gln, 10; Allopurinol.

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