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Effects of Allopurinol [4-Hydroxypyrazolo(3,4-d)Pyrimidine] on the Metabolism of Allantoin in Soybean Plants

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ABSTRACT

Some studies on the effects of xanthine oxidase inhibitor allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine] on allantoin metabolism of soybean plants (*Glycine max* cv. Tamanishiki) are reported. Soybean seedlings, aseptically germinated for 96 hours on agar containing 1 millimolar allopurinol, contained only slight amounts of allantoin, allantoic acid, and urea as compared with controls. Analysis of purines and pyrimidines of the allopurinol-treated seedlings showed marked accumulation of xanthine both in the cotyledons and seedling axes. No hypoxanthine accumulation was found. Xanthine accumulation due to allopurinol treatment was relatively low after the cotyledons had fallen. For nodulated plants, allopurinol caused a significant drop in allantoin (+allantoic acid) in the stems and nodules, accompanied by a striking accumulation of xanthine in the nodules. The xanthine concentration in the nodules far exceeded that in the germinated seedlings. Allopurinol at a concentration of 50 micromolar strongly inhibited xanthine oxidase prepared from soybean nodules.

The results suggested that the main pathway of allantoin formation in soybean plants was through purine decomposition, via xanthine-uric acid. It was specially noted that a very active purine-decomposing system existed in soybean nodules.

Allantoin is well known as one of the principal end products of nitrogen metabolism in animals and is produced by a biosynthetic sequence involving the formation of inosinic acid and its subsequent stepwise breakdown. Generally a variety of microorganisms, *e.g.* bacteria (2, 7), yeasts (30), fungi (3), and algae (1) have the ability to decompose oxypurines and consequently allantoin.

Some higher plants such as maple, comfrey, and leguminous plants also produce and accumulate large amounts of allantoin, up to half the amount of their total N (24). In contrast to the animal systems which excrete allantoin as a waste material, these plants accumulate it within cells, transport it to various organs, and further reutilize its nitrogen (24, 29). Several authors (5, 10, 17), by experiments using ^{14}C-labeled purines or glycine, have demonstrated that allantoin or allantoic acid is formed through oxidative purine decomposition by a well defined reaction sequence in animals and microorganisms. Mothes (24) and Reinbothe (29) have, however, discussed in their reviews another plausible mode of allantoin synthesis, *i.e.* direct formation from allantoic acid via condensation of urea and glyoxylic acid. This simple reverse reaction may be supported by the finding that when [^{14}C]urea was fed to banana leaves allantoic acid was heavily labeled (12), and by a report of Brunel (9) suggesting an enzymic synthesis of allantoic acid from urea and glyoxylic acid in higher fungi. While it is likely that in higher plants most allantoin and allantoic acid are formed by the same pathway as that demonstrated in microorganisms and in animal tissues, the possibility that some might be made by the condensation of urea with glyoxylic acid has not been eliminated.

Allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine] is a potent inhibitor of xanthine oxidase. Since it can prevent the formation of uric acid from xanthine or hypoxanthine it has been used to control the overproduction of uric acid in gouty subjects. While experimental use of allopurinol in animal systems has been reported (28, 36), little is known about its effect in plants. To the extent that allantoin biosynthesis in soybeans occurs via degradation of purines, application of allopurinol should result in the accumulation of xanthine or hypoxanthine instead of allantoin. Furthermore, the rate of loss of existing allantoin in the presence of allopurinol will be an indication of the ordinary rate of allantoin turnover. In the present work allopurinol has been used as a tool in this way, to examine physiological aspects of allantoin metabolism in soybean plants in which allantoin and allantoic acid play a significant role in nitrogen nutrition (14, 18, 22).

MATERIALS AND METHODS

In experiments in which the effect of allopurinol on germinating seeds was studied, soybean seeds (*Glycine max* cv. Tamanishiki) were sterilized with 0.1% copper sulfate for 10 min, washed three times with sterile water, and aseptically germinated on 0.8% agar containing 1 mM allopurinol at 30 C in the dark. After 96 hr of incubation cotyledons and seedling axes were cut from the germinated seedlings, weighed, and extracted with cold perchloric acid. In experiments in which the plants at different growth stages were used, sterile soybean seeds were germinated in Vermiculite in a growth chamber under the condition of 14 hr light and 10 hr dark at 25 to 30 C. The seedlings in Vermiculite were supplied with sterile distilled H_2O when necessary. After a period of about 2 weeks when cotyledons fall, plants were inoculated with nodule bacteria (*Rhizobium japonicum* J 5033) and thereafter supplied with nitrogen-free nutrient solution (14) every 2 days. At 4 to 5 weeks after sowing, fully developed nodules were observed on the root surfaces. Plants were harvested at 3, 10, 18, and 35 days after sowing, their roots thoroughly washed with distilled H_2O, transferred to a solution consisting of 0.01 M potassium dihydrogen phosphate and 0.5 mM allopurinol (adjusted to pH 7 with potassium hydroxide), and incubated under the same conditions as described above. The solution was aerated by bubbling throughout the incubation period.

The procedures for extraction from each tissue section were as follows: 15- to 20-g samples were homogenized for 5 min in a Waring Blendor in 50 ml of ice-cold 0.5 N perchloric acid. The homogenate was centrifuged at 10,000*g* for 10 min at 0 C. The supernatant solution was filtered and neutralized to pH 6.8 with cold 4 N potassium hydroxide. After removal of the resultant potassium perchlorate by filtration, the cleared solution was analyzed for purines and pyrimidines by ion exchange chromatography. An aliquot of this perchlorate-free solution was also used for

the quantitative analysis of allantoin, allantoic acid, and urea. Allantoin and allantoic acid were determined by the method of Trijbels and Vogels (33). For the estimation of urea the usual Conway microdiffusion method was employed. The procedures used for analysis of purines and pyrimidines were essentially the same as those reported in detail by Bonnelycke *et al.* (8), although the analytical conditions (column type, buffer timing, buffer flow rate, etc.) were somewhat different. Purines and pyrimidines were separated with the automatic system normally used for amino acid analysis, and each compound. eluted from the column (0.9 × 75 cm) packed with Amberlite CG 120, was detected in quartz flow-through cell (1 × 1 cm) by its UV absorption at 260 nm. The same buffer systems designated by Bonnelycke *et al.* (8) were employed, but the elution program was slightly modified by better separation of each peak in this column.

To examine the effect of the antibiotic azaserine, a similar experiment was carried out using 3-day-old soybean seedlings. Seedlings were incubated in a solution consisting of 0.01 M potassium dihydrogen phosphate and 1 mM azaserine over a period of 24 hr at 26 C in the dark. The seedlings were separated into cotyledons and seedling axes, weighed, and extracted with 80% ethanol. The alcohol extract obtained was subjected to amino acid analysis using an amino acid autoanalyzer. Pretreatment by ion exchange resin (a combination of anion exchanger and cation exchanger) and subsequent acid hydrolysis of amides were according to Wang (34).

Comparable plants, which were not treated with allopurinol or azaserine but grown in a same manner as treated ones, were used as control in all experiments.

For the measurement of inhibition of xanthine oxidase by allopurinol, a bacteroid fraction was prepared from root nodules of soybean plants. The bacteroids isolated from fresh nodules by the method of Bergersen (6) were suspended in 0.5 M phosphate buffer (pH 7.4) and used directly for enzyme assay. Activity of xanthine oxidase was determined by measuring the increase at 290 nm due to uric acid formed by enzymic oxidation of xanthine. The reaction mixture contained 0.5 M phosphate buffer (pH 7.4), 0.3 mM xanthine, and bacteroid suspension in a final volume of 3 ml. Incubation was carried out at 37 C for 30 min.

Allopurinol was obtained from Sigma and crystalline allopurinol riboside was a gift of T. Sakai. Azaserine was purchased from P-L Biochemicals Inc.

RESULTS

The effects of allopurinol on germinating soybean seeds are given in Table I. Allopurinol effectively repressed the accumulation of allantoin, allantoic acid, and urea in the seedling axes and cotyledons. No repression of germination or no significant difference in fresh wt between control and allopurinol-treated soybean was observed, although the concentration of allopurinol was sufficient to reduce these nitrogen compounds. This indicates that allopurinol is essentially nontoxic and the normal growth and development of the seedling can occur without allantoin and allantoic acid; in other works, these compounds are not essential for seedling growth during the early phase of germination.

The elution chromatograms of the acid extracts obtained from seedling axes and cotyledons are shown in Figure 1. Some ambiguous peaks appearing on the first portion of the elution chromatograms are the mixtures of nucleotides and other acidic UV absorbing compounds. Although some nucleosides, such as adenosine and cytosine in seedling axes, and adenosine and guanosine in cotyledons, were detected, free purines and pyrimidines were present only in trace amounts in the control seedlings, suggesting that the size of their pools was exceedingly small. Allopurinol added into an agar medium was readily incorporated within cells and caused a striking accumulation of xanthine both in the seedling axes and cotyledons during 96 hr.

Table I. Effect of Allopurinol on Fresh Weight, Allantoin, Allantoic Acid and Urea Content of Germinated Soybean Seedlings

Sterilized soybean seeds were aseptically germinated on 0.8 % agar containing 1 mM allopurinol at 30 C for 96 hr. Fresh weight, allantoin, allantoic acid and urea were determined on ten seedlings. Each value is the average of duplicate experiments.

	Fresh wt	Allantoin	Allantoic Acid	Urea
	g/10 parts	μmoles/10g Fresh wt		
Seedling axis				
Control	7.4	11.4	13.4	18.6
Allopurinol-treated	7.0	1.8	1.4	ND[1]
Cotyledon				
Control	5.3	3.2	7.7	8.6
Allopurinol-treated	5.1	ND[1]	0.9	ND[1]

[1] Not detectable.

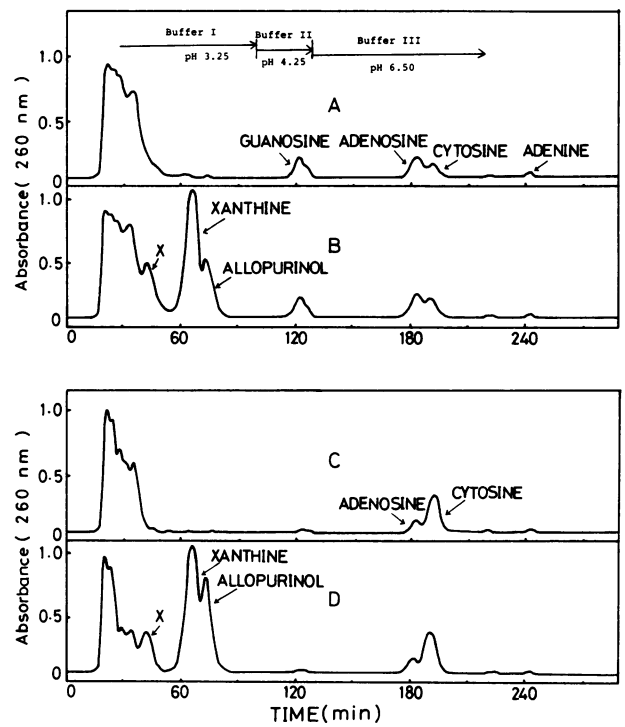

FIG. 1. Elution chromatograms of UV absorbing compounds in acid extracts of control and allopurinol-treated soybean seedlings. Experimental conditions are given in Table I. Samples were divided into cotyledons (A: control; B: allopurinol-treated) and seedling axes (C: control; D: allopurinol-treated), and extracted with 0.5 N perchloric acid by the procedures described in text. Sodium citrate-HCl buffer (pH 3.25, 4.25, and 6.50) was used for elution (1 ml/min). Buffer was changed at the time arrowed. X shown in B and D indicates UV absorbing unknown peak caused by the allopurinol treatment.

Weir and Fisher (36) reported that administration of allopurinol resulted in a marked excretion of hypoxanthine as well as xanthine by chicks. No hypoxanthine accumulation was, however, found in the soybean seedlings. This may be due to the existence of purine salvage pathway in the seedlings, including interconversion of hypoxanthine into inosine or inosinic acid, and further transformation into AMP and GMP (25). This possibility may be supported by the fact that the incorporation of [^{14}C]hypoxanthine into nucleic acids remarkably increased when allopurinol was fed to mice bearing tumors (28).

One more change caused by allopurinol treatment was the appearance of an unkown peak at a position between 40 and 50

min on the elution chromatograms of seedling axes and cotyledons. This compound was identified as allopurinol riboside by its UV absorption spectrum and by comparison of the elution time of the unknown with an authentic sample. The mechanism of the production of allopurinol riboside has been investigated by Sakai *et al.* (31) using bacteria and they found that allopurinol riboside was produced through N-ribosyl transfer reaction between pyrimidine nucleotide and allopurinol. Similar mechanism may be possible also in soybean seedlings, although no information was obtained as yet about this ribosylation reaction in plants.

All of the above data suggested that a rapid purine-decomposing system existed in germinating soybean seeds, and allantoin and allantoic acid were formed through breakdown of these purines via xanthine (and probably uric acid).

In order to examine whether these purines are produced as a result of destruction of native macromolecules (nucleic acids) in cotyledons or newly formed by *de novo* synthesis within growing cells, another experiment using the antibiotic azaserine, which interferes with purine biosynthesis, was carried out in the germinated soybean seedlings. Azaserine is a glutamine antagonist and, therefore, prevents the transamidation of glutamine amide-N to formylglycinamide ribonucleotide, an intermediate for purine biosynthesis (35). Krakoff and Karnofsky (16) showed that this inhibitor could reduce uric acid production in chickens, pigeons, and cormorants.

For this experiment, 3-day-old soybean seedlings were chosen because of the remarkable formation of allantoin in this stage. The results are shown in Tables II and III. Azaserine did not influence the fresh wt of the seedlings, although it repressed the occurrence of side roots. This inhibitor caused a 3-fold increase in glutamine. However, other amino acids were little affected except aspartic acid, glutamic acid, and proline, showing a slight decrease or increase compared with the control (Table II). The increase in glutamine content confirms that azaserine was taken up by the roots and competed with glutamine within cells. Nevertheless, no significant difference in allantoin or allantoic acid content between azaserine-treated seedlings and control was seen (Table III). This suggests that allantoin formation in germinating soybeans is not due to the decomposition of newly synthesized purines in growing cells.

A minimal metabolic rate of allantoin may be estimated from a measurement of the increase in the amount of xanthine and decrease in that of allantoin per a given time caused by allopurinol treatment. To examine this aspect of allantoin metabolism with the growth of plants, similar experiments were extended to the soybeans at different growth stages.

The effects of allopurinol on 3-, 10-, and 18-day-old soybeans are shown in Table IV. These plants are physiologically in different states; *i.e.* at the 3rd day (early germination stage), marked elongation of seedling axis occurs as a result of vigorous synthesis of protein and nucleic acids in this tissue accompanied by an increase in the catabolic activities in cotyledons; at the 10th day (late germination stage) when the cotyledons fade and the primary leaf appears, the greater part of the cotyledonous reserves are exhausted; and at the 18th day when the cotyledons fall and several trifoliate leaves appear, the plants no longer depend on the food reserves in the cotyledons and thereby autotrophic growth starts. Xanthine accumulation due to allopurinol treatment was highest in the early germination stage and slightly higher in the cotyledons than in the roots, whereas the decrease in allantoin was low. This suggests that at this stage allantoin accumulates without being utilized. In contrast, at the late germination stage xanthine accumulation was lowered, especially in cotyledons, and a striking decrease in allantoin was found. This indicates that in this stage allantoin, which had accumulated during the early germination stage, was decomposed or quickly transported to the upper regions through the stems. On the other hand, the rate of allantoin metabolism slowed at the 18th day after sowing, since both xanthine accumulation and allantoin decrease was not so prominent.

Allantoin concentration again starts to increase after nodule appearance and its level is consistently kept high throughout the reproductive stage when active nitrogen fixation is proceeding (14, 22). The effects of allopurinol on such nodulated soybean plants are shown in Figure 2 (presented as a function of incubation time). Allopurinol brought about a striking drop in allantoin present in the nodules and stems accompanied by a remarkable increase in

Table II. Effect of Azaserine on Free Amino Acid Composition of Soybean Seedling Axes

Soybean seedlings (3 days after sowing) were incubated in a solution consisting of 0.01 M KH_2PO_4 and 1 mM azaserine over a period of 24 hr at 26 C in the dark. The results listed are the average of duplicate experiments.

Amino Acids	Control	Azaserine-treated	Azaserine / Control
	μmoles/10g Fresh wt		
Asp	8.8	4.4	0.5
Asn	606.0	641.2	1.1
Thr	31.2	28.8	0.9
Ser	115.2	125.6	1.1
Glu	4.0	2.8	0.7
Gln	6.8	21.6	3.2
Pro	7.6	12.0	1.6
Gly	3.2	3.2	1.0
Ala	34.0	30.0	0.9
Val	43.2	48.8	1.1
Ile	18.0	18.0	1.0
Leu	5.2	4.4	1.1
Tyr	ND[1]	ND[1]	-
Phe	4.0	4.4	1.1
Lys	4.8	5.6	1.2
His	35.2	37.2	1.1
Arg	ND[1]	ND[1]	-
NH_3	185.0	188.7	1.0

[1] Not detectable.

Table III. Effect of Azaserine on Fresh Weight, Allantoin and Allantoic Acid Content of Soybean Seedling Axes

Experimental conditions are shown in Table II. Fresh weight, allantoin and allantoic acid were determined on ten seedlings. Each value is the average of duplicate experiments.

	Fresh wt	Allantoin	Allantoic Acid
	g/10 parts	μmoles/10g Fresh wt	
Control	5.3	12.6	27.5
Azaserine-treated	5.3	13.5	27.8

Table IV. Xanthine Accumulation and Allantoin (+ Allantoic Acid) Decrease by Allopurinol Treatment in Soybean Plants at Different Growth Stages

Soybeans grown in a growth chamber were harvested at 3, 10, 18 days after sowing and incubated in a solution consisting of 0.01 M KH_2PO_4 and 0.5 mM allopurinol for 24 hr. Each incubation vessel contained five plants. The data are the average of triplicate experiments.

Days (after sowing)		Xanthine Accumulation μmoles/10g Fresh wt/24 hr	Allantoin (+Allantoic Acid) Decrease %	Allantoin (+Allantoic Acid) Concentration μmoles/10g Fresh wt
3	Root	4.5 ± 0.3	5-17[1]	33.0 ± 1.2[2]
	Cotyledon	6.4 ± 0.4		
10	Root	3.1 ± 0.2	68-76	7.1 ± 0.4
	Cotyledon	1.8 ± 0.2		
18	Root	1.9 ± 0.2	8-24	3.7 ± 0.2
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[1] Values expressed as per cent of decrease against control plants.
[2] Concentration in control plants before allopurinol treatment.

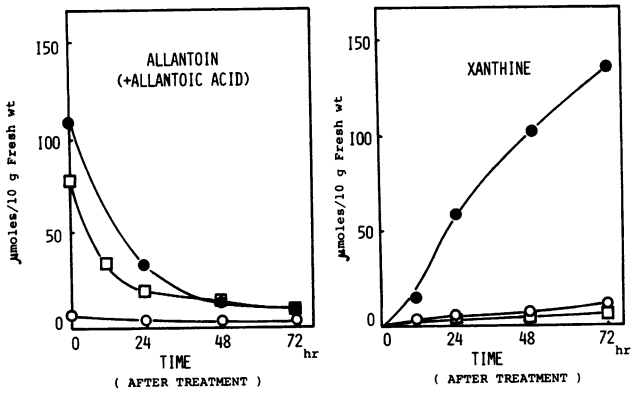

FIG. 2. Effect of allopurinol on allantoin (+allantoic acid) content and xanthine accumulation in nodulated soybean plants. Nodulated soybeans were harvested at 35 days after sowing, their roots thoroughly washed with distilled H_2O and transferred to a solution consisting of 0.01 M KH_2PO_4 and 0.5 mM allopurinol. The solution was aerated by bubbling throughout the incubation period. Each incubation vessel contained five plants. (●——●): nodule; (○——○): root; (□——□): stem.

Table V. Inhibition of Soybean Nodule Bacteroids Xanthine Oxidase by Allopurinol

Enzymatic activity was assayed as described in the materials and methods section.

Allopurinol concentration	Inhibition
μM	%
0	0[1]
1.0	25
10.0	49
50.0	75

[1] 1.55 nmol uric acid formed (37 C, pH 7.4)/min· mg protein

xanthine in the nodules. The xanthine concentration in the nodules during the first 24 hr of incubation far exceeded that in the germinated seedlings. Accumulation of xanthine was conspicuous only in the nodules and not in the roots and stems. These findings indicate that also in nodulated soybeans allantoin is formed through the purine decomposition, and furthermore that nodules are the site of its production. It was reported by Matsumoto *et al.* (21) that xylem sap of soybeans contained considerable amounts of allantoin, indicating the allantoin formation in the underground parts and its transport to the upper portions. The result shown in Figure 2, that decrease in allantoin in the stems was nearly concurrent with that in the nodules, suggests the existence of a rapid transport system carrying allantoin, produced in the nodules, to the upper portions through the stems.

In order to ascertain if this remarkable xanthine accumulation in nodules is in fact due to the competitive inhibition of xanthine oxidase with allopurinol, the effect of this inhibitor on the activity of xanthine oxidase prepared from the soybean nodule bacteroids was examined *in vitro*. The addition of allopurinol strongly inhibited oxidation of xanthine at low concentrations (Table V).

DISCUSSION

It has been shown previously that allantoin plays an important role in the translocation and storage of nitrogen in soybean plants (14). In the reproductive stage the pods of this plant contain large quantities of allantoin and allantoic acid, up to 70% of the total alcohol-soluble nitrogen (14). Mothes and his colleague (24, 29) have postulated that in such allantoin-rich plants there may be other pathways of allantoin formation in addition to the well defined route (aerobic breakdown of purines). The present investigation was undertaken in order to explore the main pathway of allantoin formation and its metabolic rate in soybean plants.

The metabolism of nucleic acid during the germination of a variety of plant species has been studied by numerous workers. Oota *et al.* (26, 27) have found that the cotyledons of bean seeds contain large amounts of RNA which accumulated during maturation of the seeds, and with the start of the germination the level of this RNA (termed storage RNA) is reduced sharply, and the drop is balanced with an increase in the growing seedling axis. According to them, the RNA reserved in bean cotyledons is broken down into small size free RNA (termed transport RNA), and this free RNA is further transported to actively growing portions or RNA sinks to be reutilized there for the reconstruction of ribosomes. A similar observation, that intact macromolecules (transport RNA) are moved into the growing regions without further destruction, was made by Ledoux *et al.* (19, 20) in barley seedlings. On the other hand, Barker and Douglas (4) reported that the RNA of pea cotyledons was degraded into soluble constituents and thereafter transported to the growing seedling axis. Essentially the same conclusion was obtained by Matsushita (23) in wheat seedlings. Our results suggest that the storage RNA is partly degraded to the purine level in soybean cotyledons via nucleotides and nucleosides, since the inhibition of xanthine oxidase by allopurinol caused a large accumulation of xanthine in cotyledons as well as in seedling axes (Fig. 1). The demonstrations of the presence of ribonuclease in various storage organs supports the breakdown of the RNA in these organs (4, 15, 19, 23). The result, that azaserine which inhibits the biosynthesis of purine nucleotides necessary for the construction of nucleic acids (35) did not influence the growth of germinated soybean seedlings (Table III), may be explained by this evidence. It may be that allantoin and allantoic acid, temporarily accumulated in the growing seedlings during the early germination period, are produced as a result of the stepwise breakdown of storage RNA which had been reserved in the cotyledons during seed maturation, *i.e.* these compounds are the decomposition products of surplus nucleotides not reutilized for the construction of RNA or DNA in growing cells. This hypothesis is compatible with the fact that allantoin (+allantoic acid) concentration gradually declined with the withering of cotyledons by the exhaustion of reserve materials (Table IV).

Our previous studies (13, 14) and investigations by other workers (18, 21, 22) showed a close correlation between nodule formation and allantoin production in soybean plants. The present study confirmed allantoin production in nodules and suggested its rapid transport to the upper portions through the stems. Bergersen (6) has reported the changes in nucleic acid and nucleotides content in soybean nodule bacteroids. Dilworth and Williams (11) also investigated the change in RNA and DNA content of bacteroids from yellow lupin nodules and found a marked fall in RNA and DNA per bacteroid during nodule development. There is, however, little information of an exact nature of nucleic acid metabolism in legume root nodules, and so whether the changes in the bacteroids RNA or DNA during nodule development correlate with allantoin formation in this tissue is still unknown. The mechanism of allantoin formation in nodules is probably more complicated than that in germinating seedlings due to its special construction consisting of host cells and bacteroids. The finding by Tajima (32) that soybean nodule uricase is quite different from seedling uricase in various chemical properties is, therefore, of special interest. Further work will be necessary for the elucidation of allantoin formation in soybean root nodules.

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