Phytochemistry, 1978, Vol. 17, pp 1239–1243. © Pergamon Press Ltd Printed in England 0031-9422/78/0801-1239$02.00/0

PROBABLE SITE OF ALLANTOIN FORMATION IN NODULATING SOYBEAN PLANTS

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(*Received* 24 *January* 1978)

Key Word Index—*Glycine max*; Leguminosae; soybean; nitrogen fixation; ammonia assimilation; allantoin formation.

Abstract—Nodulated soybean plants contain high concentration of allantoin in all parts. Excision of nodules from the roots brought about a marked decrease in allantoin. To examine the function of nodules in allantoin production, nodulated and nodule-detached soybeans were fed with $^{15}NH_3$ for 1 week. High abundance of ^{15}N was found in the amino acid-N fraction of both plants. In the root and stem of the nodulated plants, *ca* 80% of the nitrogen in this fraction was derived from the NH_3 added in the medium. Excess ^{15}N was detected also in allantoin-N fraction, but the ^{15}N content was very low in contrast to that in amino acid-N fraction. The site involved in the allantoin formation and the possible significance of its synthesis are discussed in relation to symbiotic nitrogen fixation.

INTRODUCTION

In a previous investigation [1] we suggested that allantoin, found in large amounts in various organs of soybean plants, had a significant role in nitrogen nutrition of this plant, possibly in the process of seed protein production. As allantoin was unlikely to be utilized when excess nitrogen was present in plants, it seemed that this nitrogen compound was a form of reserve nitrogen, as postulated earlier by Mothes [2]. Furthermore, the earlier observation that the accumulation of allantoin in soybeans was associated with nodule formation was confirmed [1].

The way in which nodules function in allantoin formation seems to be an interesting problem from a physiological point of view. The mechanism for the biosynthesis of allantoin and the site of its occurrence in soybean plants, however, still remain uncertain. To discover why nodulation causes allantoin accumulation, it is necessary, first, to find out the site of its production. Allantoin has been found in storage organs or xylem sap in some allantoin rich plant species [2]. Also in soybeans, it is reported that the xylem sap contained considerable amounts of allantoin [3], indicating the formation in the underground parts (roots or nodules).

A technique using ^{15}N-labelled ammonia as a tracer is thought to be valuable in searching for the site of allantoin formation, provided that the ammonia concentration does not inhibit nitrogen fixation by root nodules, since by isotopic analysis it is possible to discern the nitrogen incorporated by the roots from the external medium and the nitrogen fixed by the nodules from the air. If a given nitrogen compound was produced mainly in the roots, high incorporation of ^{15}N into this substance should be detected, and alternatively if it occurred in nodules, the abundance ratio of ^{15}N should be relatively low.

The purpose of this investigation was to explore the site involved in allantoin production of the soybean plants. Both the nodulated and nodule-detached plants were treated with a medium containing $^{15}NH_3$ for 1 week and ^{15}N incorporated into the nitrogen of amino acids and allantoin in the different organs were determined by MS.

RESULTS

To examine the effect of NH_3 added to the culture solution on the nitrogen fixing ability of soybean root nodules, acetylene reducing activities of the nodulated roots were surveyed during NH_3 treatment. From the comparison with the activities of control plants grown with N-free medium, it was found that the application of 50 ppm of NH_3-N caused 35–45% inhibition of acetylene reducing activity by root nodules during 1 week.

Amino-N and allantoin-N content of the nodulated plants (Nod-plants), treated with or without $^{15}NH_3$, and nodule-detached plants (Nod-D-plants), treated with $^{15}NH_3$, are shown in Table 1. The amino-N content in each organ of the Nod-D-plants did not show a decrease by removing nodules from the roots and its amount was rather higher than that of the nodulated ones grown with N-free medium. The excision of nodules, however, brought about a significant decrease in allantoin during 1 week, especially in the leaf and stem. On the other hand, Nod-plants contained high concentration of allantoin. Particularly in the stem and nodule, its amount was far above that of amino-N. The addition of ammonia to the Nod-plants caused a *ca* two-fold increase in amino-N content in the leaf, stem and root, but little effect was observed on the nodule amino-N. Ammonia treatment did not cause any increase in allantoin and in fact lowered its content in the nodule and stem. Application of ammonia caused the allantoin content to decrease *ca* 40% in the nodule. This evidence suggests that the ammonia absorbed by the roots from the culture solution represses the synthesis of allantoin, whereas it is efficiently utilized for synthesis of amino acids.

Table 1. Amino-N and allantoin-N content in nodulated and nodule-detached soybeans

	Amino-N			Allantoin-N		
	N-0* (Nod)	N-50† (Nod) (mg N/g dry wt)	N-50‡ (Nod-D)	N-0* (Nod)	N-50† (Nod) (mg N/g dry wt)	N-50‡ (Nod-D)
Leaf	0.42	0.70	0.55	0.82	1.10	0.09
Stem	0.63	1.29	1.13	4.21	3.68	0.29
Root	0.51	0.95	0.89	1.11	1.08	0.48
Nodule	1.62	1.54		3.01	1.79	

* Nodulated soybeans grown with N-free medium.
† Nodulated soybeans applied with 50 ppm of NH_3-N during 1 week.
‡ Nodule-detached soybeans applied with 50 ppm of NH_3-N during 1 week.

Table 2 presents the atom % excess ^{15}N in EtOH soluble-N and EtOH insoluble-N fraction of the Nod-plants and Nod-D-plants. The incorporations of ^{15}N into both fractions of Nod-D-plants, were lower than those of the nodulated ones, despite their dependence for nitrogen source entirely on the externally added $^{15}NH_3$. The low incorporation of ^{15}N found in the Nod-D-plants reflects the depressed growth of these plants. It seems likely that removal of nodules from the roots influenced significantly the whole metabolism of these plants, not only in nitrogen metabolism. The reason for the striking decrease in allantoin by nodule excision (Table 1) is probably because allantoin production almost stops and allantoin already present decomposes to provide nitrogen for the synthesis of other essential compounds. This is due to the nitrogen deficiency caused by the lowering of the nitrogen absorbing ability of the roots and the stoppage of the fixed nitrogen supply from nodules. As to the Nod-plants, the abundance of ^{15}N in the nodule was lower than that in other organs, indicating that the nodule itself is dependent for its metabolism almost entirely on the fixed nitrogen from the air.

The data for the incorporation of $^{15}NH_3$ into amino acid-N and allantoin-N fraction are given in Table 3 and Fig. 1 (Fig. 1 illustrates the content of ^{15}N derived from $^{15}NH_3$ in the culture solution). Although the amino-N content of Nod-D-plants was the same as that of the nodulated ones as shown in Table 1, ^{15}N incorporated into the amino acid-N fraction of these plants was considerably lower than that of the Nod-plants. It is assumed that the major part of the amino acids present in the Nod-D-plants are not newly synthesized ones from the nitrogen absorbed by the roots. The results of the amino acid analysis of the Nod-plants and Nod-D-plants (Table 4) may support this, since in the Nod-D-plants the proportion of such amino acids as were present in only minute amounts in the Nod-plants increased (e.g. leucine, tyrosine, histidine, arginine, etc.), suggesting that these minor amino acids were increased due to the decomposition of the native proteins.

In contrast to the Nod-D-plants, high incorporation of ^{15}N into the amino acid-N fraction was detected in the Nod-plants. In the stem and root, *ca* 80 % of the nitrogen

Table 2. Atom % excess ^{15}N in alcohol insoluble-N fraction and alcohol soluble-N fraction

	Nodulated soybeans		Nodule-detached soybeans	
	Alcohol insoluble-N (%)*	Alcohol soluble-N (%)	Alcohol insoluble-N (%)	Alcohol soluble-N (%)
Leaf	2.42	3.06	0.41	1.10
Stem	3.25	4.33	0.72	2.39
Root	2.93	4.92	1.24	3.36
Nodule	0.12	0.35		

* Atom % excess ^{15}N.

Table 3. Atom % excess ^{15}N in amino acid-N fraction and allantoin-N fraction

	Nodulated soybeans		Nodule-detached soybeans	
	Amino acid-N (%)*	Allantoin-N (%)	Amino acid-N (%)	Allantoin-N (%)
Leaf	4.87	0.84	2.23	0.80
Stem	8.19	1.29	2.92	2.44
Root	8.58	1.21	4.52	2.32
Nodule	1.39	0.25		

* Atom % excess ^{15}N.

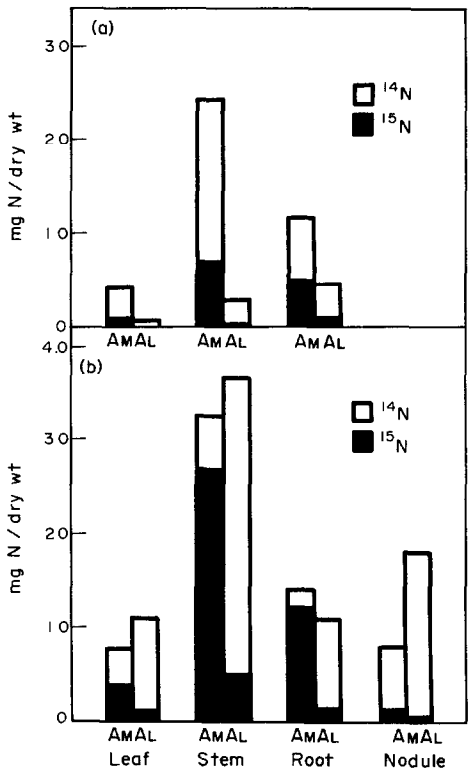

Fig. 1. Incorporation of ^{15}N into amino acids-N fraction and allantoin-N fraction: (a) nodule-detached soybean plants; (b) nodulated soybean plants. AM and AL express amino acid-N and allantoin-N, respectively. Amino acid-N fraction includes α-amino-N of each of amino acid, and amide-N of asparagine and glutamine. ^{15}N shaded indicates the nitrogen derived from NH_3 in a medium.

in this fraction was derived from the externally added $^{15}NH_3$ (Fig. 1b). Furthermore, the amino acid analysis indicated that the amide asparagine occupied a significant proportion of this fraction (Table 4). The excess ^{15}N was detected in the amino acid-N of the nodule as well, but not appreciably as compared with other parts, showing that the amino acids in nodules are largely synthesized from the biologically fixed nitrogen in this tissue. Whether the amino acids labelled with ^{15}N in nodules are the transported ones from the roots or the synthesized ones in the nodule is, however, unknown. The high abundance of ^{15}N found in the root amino acid-N fraction suggests a rapid assimilation of ammonia to amino acids in this tissue, and the fact, that the abundance ratio in the stem was similar to that in the root, indicates that amino compounds synthesized in the roots are immediately carried to the upper regions through the stem. It must be specially noted that the contribution of fixed nitrogen from the air to the amino acids present in the host plants was unexpectedly small when 50 ppm of NH_3-N was applied to the culture solution.

As for the allantoin-N fraction, excess ^{15}N was also detected in the Nod-D-plants whose allantoin content strikingly decreased during 1 week after the removal of nodules, suggesting a contribution of the ammonia absorbed by the roots to allantoin formation. ^{15}N content in this fraction was, however, considerably lower than that in the amino acid-N fraction as shown in Fig. 1a. ^{15}N was incorporated into the allantoin-N fraction of the allantoin-rich Nod-plants, too, but the atom % excess showed very low values as compared with that of the amino acid-N fraction. The lowest atom % excess ^{15}N of this fraction was found in the nodule. This evidence clearly suggests that a large share of allantoin-N in the nodulated soybeans is derived from the biologically fixed nitrogen supplied by the root nodules.

Table 4. Amino acid composition of nodulated and nodule-detached soybeans

	μmol/g dry wt						
	Leaf		Stem		Root		Nodule
	Nod*	Nod-D†	Nod	Nod-D	Nod	Nod-D	Nod
Asp	6.5	3.2	5.2	9.2	2.4	2.9	5.1
Asn	9.6	6.4	102.6	70.3	42.5	31.4	13.3
Thr	1.6	1.0	1.2	‡	0.6	1.7	1.2
Ser	6.2	4.5	6.5	6.5	2.6	3.0	4.4
Glu	8.5	3.4	3.3	1.5	1.8	2.6	4.8
Gln	1.3	0.9	2.0	0.9	2.0	1.7	0.7
Pro	‡	0.6	3.7	‡	‡	‡	‡
Gly	1.1	0.6	‡	0.4	0.6	0.6	0.6
Ala	5.1	2.2	2.9	1.6	2.2	1.9	5.0
Val	0.3	0.4	‡	1.3	0.3	0.5	‡
Met	‡	‡	‡	‡	‡	‡	‡
Ile	0.3	0.5	0.4	2.0	0.3	0.5	0.2
Leu	0.2	0.5	‡	1.3	0.2	0.6	0.2
Tyr	‡	0.7	‡	0.4	‡	0.1	‡
Phe	0.6	1.7	‡	1.4	‡	0.6	‡
Lys	0.8	0.4	‡	0.4	0.3	0.4	‡
His	0.9	1.5	‡	1.5	0.5	1.7	2.7
Arg	1.3	2.3	‡	4.5	‡	0.6	3.3

* Nodulated soybean plants.
† Nodule-detached soybean plants.
‡ Not detectable.

DISCUSSION

It has been suggested that the degree of nodulation influences the allantoin content in soybeans [1, 4]. This was shown from the result that the allantoin content in well-nodulated soybeans grown without nitrogen fertilizer was always higher than that in beans applied with large amounts of nitrogen fertilizer, consequently inferior in nodule number. A close correlation between nodule formation and allantoin accumulation was reinforced by the fact that allantoin was present only in a small quantity in non-nodulating soybeans, a strain genetically incapable of nodulation [1, 4].

For the function of nodulation in allantoin production, two interpretations may be considered. One is that allantoin formation in root cells is stimulated by the nodules, and the other is that the major part of allantoin is formed preferentially in the nodules. There are many reports, up to now, that various plant hormones stimulate nucleic acid metabolism in plant cells [5]. As allantoin is known to be formed through purine breakdown in microorganisms and animal systems, it is possible to suppose that the nodules have a hormonal effect on the roots by which a rapid turnover of the nucleic acids and consequently over production of allantoin is brought about. The finding by Phillips and Torrey [6] that cytokinin was released into the medium by cultures of *Rhizobium japonicum* or *R. leguminosarum* may support this explanation. The result that excess ^{15}N was detected in the allantoin-N fraction of the nodule-detached plants indicates that the root by itself is capable of producing allantoin, although the rate of its synthesis is very low. If the possibility that nodules promote allantoin formation in the roots is right, in the allantoin-rich nodulated soybeans ^{15}N content in the allantoin-N fraction should be higher than that in the nodule-detached ones. The results, however, showed that the ^{15}N content in the allantoin-N fraction of the nodulated soybeans was never high in contrast to the allantoin content of this plants (Fig. 1b), and hence the abundance ratio of ^{15}N in this fraction was lower than that of the nodule-detached ones (Table 3). These data suggest that the nodules do not promote allantoin formation in roots. The evidence, that $^{15}NH_3$ absorbed by the roots from outside is not so much utilized in the synthesis of allantoin, while utilized efficiently in a synthesis of amino acids, largely amide asparagine, makes the possibility that the allantoin is formed in the nodules more likely. It is reasonable to suppose that the greater part of the allantoin is produced in nodule tissues and immediately transported to the other portions. The data from Table 1, showing that those plants grown with nitrogen-free medium, therefore depending for growth completely on the biologically fixed nitrogen, contained high concentrations of allantoin in all parts, are well explained by this.

Tajima [7] reported the nature and localization of soybean uricase, which catalyzes the reaction of oxidative decomposition of uric acid into allantoin, and found high uricase activity in nodules, but not in other organs. Furthermore, in recent investigations we have shown that when $^{15}N_2$ and $^{15}NH_3$ was supplied to the excised nodules or nodule homogenates ^{15}N was incorporated into the nitrogen in the allantoin molecule (in preparation). This evidence strongly suggests the existence of allantoin biosynthetic pathways in nodules.

It has been established that NH_3 is the primary stable intermediate in symbiotic nitrogen fixation, and that other nitrogen compounds such as glutamic acid and glutamine are formed from it. Convincing evidence for this has been provided by many workers, with the experiments in which $^{15}N_2$ gas was exposed to the root nodules [8–12]. The highest enrichment of ^{15}N in glutamic acid and glutamine observed has been explained by the finding of high activities of enzymes such as glutamate dehydrogenase, glutamate synthase, and glutamine synthetase in various nodules [13–17]. Of these enzymes, glutamine synthetase is usually found in large amounts in the nodule cytosol fraction from various sources [14, 16, 17]. Miflin and Lea [18] have suggested that the great excess of glutamine synthetase in the nodule cytosol could be of key importance in the nitrogen-fixing symbiotic relationship. Despite the extremely high level of this enzyme in nodules, the glutamine content in soybeans was very low in every organ as shown in Table 4. This means that glutamine is rapidly metabolized in nodules without being pooled or transported, probably utilized for the synthesis of other nitrogen compounds. Provided that allantoin formation in nodules is due to the oxidative decomposition of purines, a role of glutamine as an important precursor for allantoin biosynthesis will be possible, since the nitrogen atoms at position 3 and 9 in the purine ring are known to be derived from glutamine amide nitrogen. It seems likely, assuming that this is true, that allantoin formation has a special role in the ammonia assimilation process of soybean nodules.

EXPERIMENTAL

Plant growth and $^{15}NH_3$ *treatment.* Soybean plants (*Glycine max* cv Tamanishiki) were grown in vermiculite applied with a mineral nutrient soln lacking N [1] in a greenhouse over 5 weeks. All plants were inoculated with an efficient strain of *Rhizobium japonicum*. The nodulated soybeans obtained were further grown with a N-free medium described above or a medium containing 50 ppm of $^{15}NH_3$-N in the form of $(NH_4)_2SO_4$ (10 atom % excess ^{15}N) for 1 week. Nodule-detached plants, where nodules were all excised from the roots by hand, were also grown with the same soln applied 50 ppm of $^{15}NH_3$-N. All culture solns were renewed every 2 days. After $^{15}NH_3$ treatment both nodulated and nodule-detached plants were harvested and divided into roots, stems, leaves and nodules. Each organ was frozen at $-20°$, dried in *vacuo* and subjected to quantitative analysis of allantoin-N and amino-N, or a determination of ^{15}N enrichment in each fraction.

EtOH *soluble*-N *and* EtOH *insoluble*-N *fraction.* Each sample was extracted with hot 80% EtOH and the insoluble material was removed with a filter paper. The resultant filtrate and the debris remaining on filter paper are referred to as the EtOH soluble-N and EtOH insoluble-N fraction respectively. An aliquot of the EtOH soluble-N fraction was used for the quantitative analysis of amino-N [19] and allantoin-N [20], and the other portion of this fraction was subjected to isotopic analysis. The EtOH insoluble-N was converted to NH_3 by Kjeldahl digestion. The EtOH soluble-N fraction was evaporated to dryness to remove EtOH and the resulting residue was subjected to digestion in the same manner. Digested samples were then steam distilled and the NH_3 evolved was collected in 0.02 N H_2SO_4.

Amino acid-N *fraction.* A separation of amino acids and allantoin was accomplished with ion exchange resins. Amino acids held with Dowex 50 (H^+) were eluted with 6 N HCl. The eluate was then adjusted to 3 N with H_2O and heated at 100° for 1 hr to hydrolyze amides. This fraction, termed amino acid-N fraction, contains both α-amino-N, and NH_3-N re-

leased from amides. α-Amino-N of this fraction was released as NH_3 by the specific reaction with ninhydrin [21] and recovered by steam distillation. As for each of the amino acids, their separation was according to Wang [22] and their quantitative analysis was carried out in an automatic amino acid analyzer.

Allantoin-N fraction. The conversion of allantoin to NH_3 included 3 steps: alkaline hydrolysis of allantoin to allantoic acid, acid hydrolysis of allantoic acid to urea and enzymic decomposition of urea to NH_3. The procedures used were as follows. EtOH extract was adjusted to pH 12 with satd $Ba(OH)_2$ soln and then heated at 100° for 20 min to hydrolyze allantoin. After cooling, Ba^{2+} was precipitated by H_2SO_4 and the resulting $BaSO_4$ was removed with centrifugation. This supernatant was poured on a Dowex 1 ($HCOO^-$) column. Allantoic acid adsorbed to this column was eluted with 2 N HCOOH. Allantoic acid is weak in acid soln [23] and so further decomposes into urea and glyoxylic acid moieties during the elution with HCOOH. After removing of HCOOH with a rotary evaporator the residue was dissolved in 0.1 M Pi buffer (pH 7) and used in the following enzymic decomposition of the resultant urea. Conversion of urea to NH_3 was performed by the usual Conway's micro-diffusion method.

^{15}N *analysis.* Estimation of NH_3 in each fraction was made by Nessler's reagent. For ^{15}N analysis NH_3 was further converted to N_2 gas with NaOBr. The abundance of ^{15}N in N_2 gas was determined by MS. For the calculation of the atom % excess ^{15}N, a value of 0.370 atom % was employed as the natural abundance.

REFERENCES

1. Fujihara, S., Yamamoto, K. and Yamaguchi, M. (1977) *Plant and Soil* **48**, 233.
2. Mothes, K. (1961) *Can. J. Botany* **39**, 1785.
3. Matsumoto, T., Yamamoto, Y. and Yatazawa, M. (1976) *J. Sci. Soil Manure, Japan* **47**, 463.
4. Kushizaki, M., Ishizuka, J. and Akamatsu, F. (1964) *J. Sci. Soil Manure, Japan* **35**, 323.
5. Key, J. L. (1969) *Ann. Rev. Plant Physiol.* **20**, 449.
6. Phillips, D. A. and Torrey, J. G. (1972) *Plant Physiol.* **49**, 11.
7. Tajima, S. and Yamamoto, Y. (1975) *Plant Cell Physiol.* **16**, 271.
8. Aprison, M. H., Magee, W. E. and Burris, R. H. (1954) *J. Biol. Chem.* **208**, 29.
9. Gardner, I. C. and Bond, G. (1958) *J. Exp. Botany* **9**, 320.
10. Leaf, G., Gardner, I. C. and Bond, G. (1959) *Biochem. J.* **72**, 662.
11. Bergersen, F. J. (1965) *Aust. J. Biol. Sci.* **18**, 1.
12. Kennedy, I. R. (1966) *Biochim. Biophys. Acta* **130**, 285.
13. Nagatani, H., Shimizu, M. and Valentine, R. C. (1971) *Arch. Mikrobiol.* **79**, 164.
14. Dunn, S. D. and Klucas, R. V. (1973) *Can. J. Microbiol.* **19**, 1493.
15. Ryan, E. and Fottrell, P. F. (1974) *Phytochemistry* **13**, 2647.
16. Brown, C. M. and Dilworth, M. J. (1975) *J. Gen. Microbiol.* **86**, 39.
17. Robertson, J. G., Warburton, M. P. and Farnden, K. J. F. (1975) *FEBS Lett.* **55**, 33.
18. Miflin, B. J. and Lea, P. J. (1976) *Phytochemistry* **15**, 873.
19. Yemm, E. W. and Cocking, E. C. (1955) *Analyst* **80**, 209.
20. Trijbels, F. and Vogels, G. D. (1966) *Biochim. Biophys. Acta* **113**, 292.
21. Kennedy, I. R. (1965) *Anal. Biochem.* **11**, 105.
22. Wang, D. (1968) *Contrib. Boyce Thompson Inst.* **24**, 109.
23. Young, E. G. and Conway, C. F. (1942) *J. Biol. Chem.* **142**, 839.