J. Biosci., Vol. 6, Number 2, June 1984, pp. 185–192.

Ureide biogenesis and the enzymes of ammonia assimilation and ureide biosynthesis in nitrogen fixing pigeonpea (*Cajanus cajan*) nodules

AMARJIT and RANDHIR SINGH
Department of Chemistry and Biochemistry, Haryana Agricultural University, Hissar 125 004, India

MS received 15 November 1983; revised 24 January 1984

Abstract. Allantoic acid production from IMP, XMP, inosine, xanthosine, hypoxanthine, xanthine, uric acid and allantoin was investigated by incubating each of these substrates with *Cajanus cajan* cytosol and bacteroid fractions separately in the presence and absence of NAD^+ and allopurinol. Allantoic acid synthesis by bacteroid fraction could only be observed with uric acid and allantoin as substrates. Addition of NAD^+ or allopurinol to the reaction mixtures had no effect. However, with cytosol fraction, allantoic acid was produced by each of these substrates, with maximum rate with allantoin. With NAD^+ or with allopurinol, allantoic acid was produced only with uric acid and allantoin as substrates. NADH production with cytosol fraction could again be observed with all the substrates. Except with uric acid and allantoin, allopurinol completely inhibited NADH formation. Regardless of the presence or absence of allopurinol, none of the substrates exhibited significant activity with bacteroid fraction. Based on the activities of glutamine synthetase, glutamate synthase, glutamate dehydrogenase, aspartate aminotransferase, asparagine synthetase, nucleotidase, nucleosidase, xanthine dehydrogenase, uricase and allantoinase and their intracellular localisation in various nodule fractions, a probable pathway for the biogenesis of ureides in pigeonpea nodules has been proposed.

Keywords. Pigeonpea; *Cajanus cajan;* nodules; ureide biogenesis; intracellular location.

Introduction

In many of the tropical legumes including pigeonpea, most of the nitrogen fixed in nodules is translocated in the form of ureides, allantoin and allantoic acid (Herridge *et al.*,1978; Pate *et al*., 1978; Streeter, 1979; McClure and Israel, 1979; Luthra *et al*., 1981; Sheoran *et al*., 1982). These ureides are synthesised *via* a pathway involving biosynthesis of purines followed by oxidation and hydrolysis (Atkins *et al.*, 1980a, Schubert, 1981; Boland and Schubert, 1982). Studies on enzymes of purine biosynthesis in soybean nodules have demonstrated the presence of phosphoribosylpyrophosphate synthetase (EC 2.7.6.1), phosphoribosyl amidotransferase (EC 2.4.2.14), phosphoglycerate dehydrogenase (EC 1.1.1.95), serine hydroxymethyltransferase (EC 2.1.2.1) and 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) (Schubert, 1981; Reynolds *et al*., 1982a), indicating that purine biosynthesis in nodules parallels known pathways in animals and microorganisms. This assumption gained support when the specific activity of all these enzymes were found to increase markedly in soybean nodules during the onset of nitrogen fixation and ureide production (Reynolds *et al.*, 1982a). Moreover, their levels in soybean nodules were much greater than in nodules of lupin and pea, legumes, in which the main products of ammonium assimilation are amino

acid amides rather than ureides (Reynolds *et al.,* 1982b; Christensen and Jochimsen, 1983). Evidences also became available which indicated that purines synthesised *de novo* in the plant cells, were precursors for ureides in the nodules (Triplett *et al.*, 1980; Woo *et al*., 1980, 1981; Atkins *et al.*, 1980b; Boland and Schubert, 1982). However, the mechanism for the assimilation of fixed N into ureides still remains to be clearly elucidated. In our recent studies (Luthra *et al*., 1983a, b), we proposed a metabolic pathway for the assimilation of ureides into seed proteins in pigeonpea. In this communication, we propose a probable route for the formation of ureides from nucleotides in nodule cytosol fraction. Activities of various enzymes relative to acetylene reduction activity (N_2 fixation) are also given in an attempt to demonstrate that these enzymes may be involved in amide synthesis and ureide biogenesis in nodules of pigeonpea plants.

Materials and methods

Plant material

The crop of pigeonpea (*Cajanus cajan* L.) cv. UPAS-120, was raised in sand culture in earthen pots under natural conditions of light and humidity as described earlier (Sheoran *et al*, 1982). At weekly intervals, each pot was supplied with 250 ml of N-free nutrient solution (Wilson and Reisenaur, 1963). On other days, the pots were irrigated with tap water. Nodules from 40 to 45 day old plants were harvested and used immediately.

Preparation of cytosol, bacteroid and proplastid fractions

Cytosol fraction from freshly harvested nodules was prepared essentially according to Herridge *et al.* (1978). Bacteroids were isolated according to the method of Triplett *et al.* (1980) and proplastids were separated by following the procedure of Boland and Schubert (1983). Freshly harvested nodules (3 g) were broken in 10ml 0·1 M Tricine buffer (pH 8·0), containing 0·4 M sucrose, l0 mM KCl, 10 mM EDTA, 5mM dithiothreitol, 1 mM $MgCl_2$ and 2 mM reduced glutathione, and filtered through cheese cloth. The brei obtained was centrifuged at 1650 *g* for 4 min. The darker red fluid obtained above the bacteroids was removed carefully and layered on a step gradient comprising a 5 ml band of 0.1 M Tricine-KOH (pH 8·0) containing 0·8 M sucrose, 2 mM DTT, 2 mM reduced glutathione, and 10 mM KCl, and a second 10 ml band of 2 M sucrose in 0·1 M Tricine-KOH (pH 8·0). The step gradient was centrifuged at 9000 *g* for 30 min in a swinging bucket rotor (Beckman Ultracentrifuge). The pellicle at the interface between the two sucrose layers was withdrawn and referred to as the proplastid fraction. The purity of the three fractions was monitored by assaying the marker enzymes.

Enzyme assays

Nitrogenase (EC 1.18.2.1) activity in freshly harvested nodulated roots was determined

as described earlier (Luthra *et al*., 1983b). Other enzymes were assayed separately in each fraction. Glutamine synthetase (EC 6.3.1.2), glutamate dehydrogenase (EC 1.4.1.4), uricase (EC 1.7.3.3) and allantoinase (EC 3.5.2.5) were assayed as described previously (Sheoran *et al*., 1981). Glutamate synthase (EC 1.4.1.14) was assayed as described by Boland *et al.* (1978). Asparagine synthetase (EC 6.3.1.1) and aspartate aminotransferase (EC 2.6.1.1) were assayed by following the procedures of Rogenes (1975) and Bergmeyer and Bernt (1973), respectively. Xanthine dehydrogenase (EC 1.2.1.37) was measured by the rate of reduction of NAD^+ in the presence of xanthine (Triplett *et al*., 1982). Nucleotidase (EC 3.1.3.5) was assayed by measuring the release of inorganic phosphate from XMP. The assay mixture in a final volume of 1·5 ml contained: l00 mM Tris-maleate buffer (pH 5·3), 12 mM $MgCl_2$, 3 mM XMP, 1 mg bovine serum albumin and enzyme preparation. The mixture was incubated at 37°C for 15 min and the reaction terminated by adding 1 ml of 10% trichloroacetic acid. The precipitated protein was removed by centrifugation and the inorganic phosphate estimated in the supernatant by the method of Ozbun *et al.* (1973). Nucleosidase (EC 3.2.2.1) was assayed by coupling the activity of XDH present in the enzyme preparation and following NADH production spectrophotometrically at 340 nm (Triplett *et al*., 1982).

The marker enzymes phosphoenolpyruvate carboxylase (EC 4.1.1.31) for cytosol fraction, *β*-hydroxybutyrate dehydrogenase (EC 1.1.1.30) for bacteroid fraction and phosphoglycerate dehydrogenase for proplastid fraction were assayed according to the procedures of Hatch (1972), Wong and Evans (1971) and Boland and Schubert (1983), respectively.

Allantoic acid production from the different substrates used was measured as described earlier (Luthra *et al*., 1983b). Protein after trichloroacetic acid precipitation was determined according to Lowry *et al.* (1951).

Results and discussion

Ureide synthesis was assayed in bacteroid and cytosol fractions of pigeonpea nodules using several suspected precursors of allantoic acid synthesis. Allantoic acid could be produced from IMP, XMP, inosine, xanthosine, hypoxanthine, xanthine, uric acid and allantoin by cytosol fraction in the presence of NAD^+ (table 1). The maximum production was observed with allantoin followed by uric acid, xanthosine, inosine, hypoxanthine, xanthine, XMP and IMP. Without NAD^+ or with allopurinol, allantoic acid was produced only with uric acid or allantoin as substrate. The rate was again higher with allantoin than with uric acid. Allantoic acid synthesis by bacteroid fraction could only be observed with uric acid and allantoin as substrates. Addition of NAD^+ or allopurinol to the reaction mixtures had no effect. With this fraction also, more allantoic acid was produced when allantoin was used as the substrate than with uric acid.

Using the same substrates, NADH production was followed spectrophotometrically at 340 nm. With cytosol fraction, rate of NADH production was linear for upto 20 min (figure 1) and maximum production was observed when hypoxanthine was used as the substrate. Low rates were obtained with IMP, XMP, uric acid or allantoin as substrates

Table 1. Allantoic acid production by cytosol and bacteroid fractions of pigeonpea nodules.

Substrate	p mol/mg protein/min					
	Cytosol			Bacteroid		
	$-NAD^+$	$+NAD^+$	+ Allopurinol	$-NAD^+$	$+NAD^+$	+ Allopurinol
IMP	0	108	0	0	0	0
XMP	0	125	0	0	0	0
Inosine	0	154	0	0	0	0
Xanthosine	0	178	0	0	0	0
Hypoxanthine	0	136	0	0	0	0
Xanthine	0	133	0	0	0	0
Uric acid	266	277	269	63	64	73
Allantoin	426	434	305	161	177	141

Reaction mixtures contained 0·2 mM substrate. 2·5 mM NAD^+ and the cytosol or bacteroid fraction containing about 1 mg protein. Allopurinol (0·2 mM) was added only to those reaction mixtures which contained NAD^+. The mixtures were incubated at 30°C for upto 3 h and allantoic acid formed was estimated.

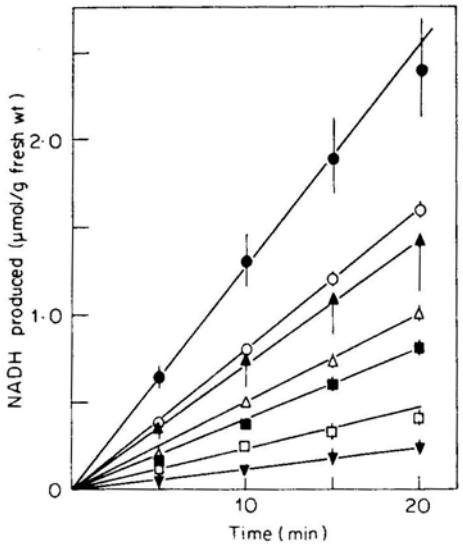

Figure 1. Time course of NADH formed by nodule cytosol fraction from various intermediates of ureide biosynthetic pathway. All assays included 2·5 mM NAD^+, 0·2 mM sub strate, 0·1 M potassium phosphate buffer (pH 7·8) and cytosol fraction containing about 1 mg protein.
(●), Hypoxanthine; (o)xanthine; (Δ), inosine; (▲) IMP; (□), XMP; (■), uric acid; (▼), allantoin.

(table 2). For all substrates except uric acid and allantoin, allopurinol completely inhibited NADH formation. Very little NADH was produced when uric acid and allantoin were used as substrates with cytosol fraction, and allopurinol had no effect on this synthesis. Regardless of the presence or absence of allopurinol, none of the substrates exhibited significant activity with the bacteroid fraction.

Results included in table 1 indicate that no substrate except uric acid and allantoin was utilised by bacteroid fraction for the formation of allantoic acid. Presence of NAD^+ or allopurinol also had no effect indicating that bacteroid is not the site for ureide synthesis. These results are contrary to those reported earlier by Tajima and

Table 2. NADH production by cytosol and bacteroid fractions of pigeonpea nodules from various intermediates of ureide biosynthetic pathway

Substrate	n mol NADH produced mg^{-1} protein min^{-1}			
	Cytosol		Bacteroid	
	−Allopurinol	+Allopurinol	−Allopurinol	+Allopurinol
IMP	2·5	0·2	0·0	0·0
XMP	1·4	0·0	0·0	0·0
Inosine	3·2	0·0	0·2	0.1
Xanthosine	5·0	0·2	0·6	0·2
Hypoxanthine	7·9	0·2	0·5	0·2
Xanthine	5·0	0·2	0·4	0·3
Uric acid	1·1	1·0	0·0	0·0
Allantoin	0·7	0·6	0·4	0·4

Reaction mixtures contained 0·2 mM substrate, 2·5 mM NAD and the cytosol or bacteroid fraction containing about 1 mg protein. NADH production was followed spectrophotometrically at 340 nm for 5 min at 25°C.

Yamamoto (1975), Tajima *et al.* (1977) and Fujihara and Yamaguchi (1978), where they proposed bacteroid as the probable site of ureide synthesis with xanthine oxidase as the xanthine oxidising enzyme. However, no xanthine oxidising activity could be detected in the present case in the bacteroid fraction and this prevented allantoic acid production by all substrates except uric acid and allantoin. This was further confirmed when no NADH production was observed in bacteroid fraction in the presence of any of the substrates. Recent studies by Hanks *et al.* (1981) have indicated that uricase and allantoinase are associated with peroxisomes and microsomal fraction of soybean nodules, respectively. The presence of uricase and allantoinase in the bacteroid fraction observed here might have thus arisen due to the contamination of bacteroid fraction by these fractions in the preparation of bacteroids. Triplett *et al.* (1980) also observed some of the activity of uricase and allantoinase to be present in bacteroid fraction mainly due to contamination.

The results included in tables 1 and 2 further indicate that ureides arise mainly in cytosol fraction. Since in the absence of NAD^+ or presence of allopurinol, none of the substrates except uric acid and allantoin could produce allantoic acid or NADH, cytosol ureide synthesis from the products of *de novo* purine biosynthesis require xanthine dehydrogenase activity. This was confirmed by the observation that the presence of allopurinol or absence of NAD^+ completely blocked the pathway. However, allantoic acid production from uric acid and allantoin was unaffected by NAD^+ or allopurinol, indicating that oxidation of these substrates followed xanthine oxidation.

To further supplement these results, various enzymes involved in ammonium assimilation and ureide biosynthesis were assayed in nodule cytosol fraction and their activities expressed relative to the activity of nitrogenase (figure 2). The results clearly indicate that all the enzymes required for the initial assimilation of ammonium into glutamine and other amino acids, as well as for the biosynthesis of ureides from the products of purine synthesis were present in quite high amounts in cytosol fraction.

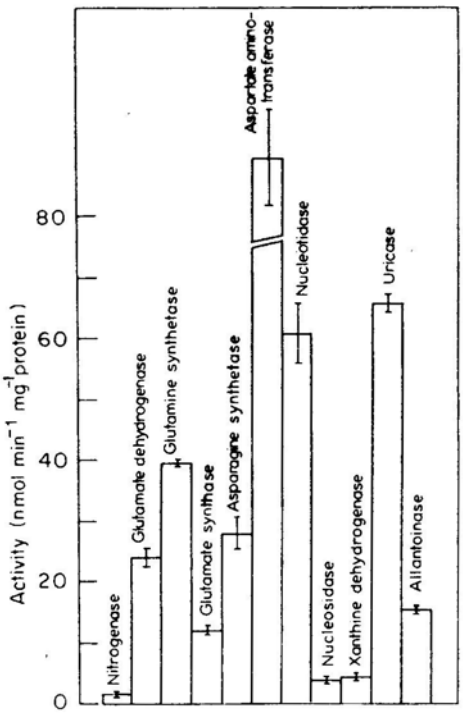

Figure 2. Enzyme activities in cytosol fraction of pigeonpea nodules relative to nitrogenase activity.

Based on all these observations, a probable pathway for biosynthesis of ureides in pigeonpea nodules could be visualised as follows: Atmospheric nitrogen is reduced to the level of ammonium by bacteroid nitrogenase. This ammonium is then transported across the bacteroidal membrane and is converted to glutamine in the cytosol by glutamine synthetase. Glutamate synthase, aspartate amino transferase and asparagine synthetase may be involved in the synthesis of amino acids and amides particularly glutamate, aspartate and asparagine etc. Glutamine and aspartate etc are then utilised for the biosynthesis of purines by well known reactions. The end product of purine biosynthesis, IMP, may be converted to XMP, by IMP dehydrogenase. This enzyme has recently been detected in soybean nodule cytosol by Shelp and Atkins (1983). IMP or XMP may be dephosphorylated by a nucleotidase to inosine or xanthosine, respectively. These could then be converted to hypoxanthine and xanthine by the enzyme nucleosidase. We could detect the activity of nucleotidase and nucleosidase in the cytosol fraction, but the activity of nucleosidase was comparatively much less than that of nucleotidase. Hypoxanthine and xanthine could then be oxidised to uric acid by xanthine dehydrogenase. Uric acid and allantoin in turn are oxidised by uricase and allantoinase, respectively to the end product, allantoic acid. All these enzymes were detected in the nodule cytosol.

To obtain information about the probable intracellular localisation of these enzymes, we isolated bacteroidal, proplastid and cytosol fractions from pigeonpea nodules by differential centrifugation and sucrose density gradient method. The purity of these fractions was examined by marker enzymes *i. e.* phosphoglycerate de-

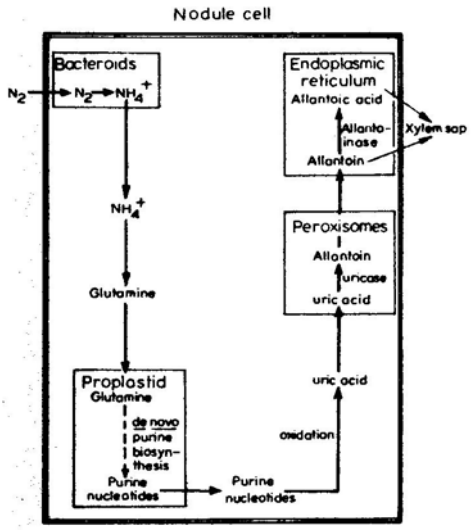

Figure 3. Proposed ureide biosynthetic reactions and their probable site of localisation in pigeonpea nodules.

hydrogenase for proplastid; *β*-hydroxybutyrate dehydrogenase for bacteroid; and phosphoenolpyruvate carboxylase for cytosol. The results of this experiment gave interesting information in that glutamine synthetase was localised mainly in cytosol and was absent from proplastid and bacteroidal fractions. Similarly, the enzymes of ureide biosynthesis from purines *i.e.* nucleotidase, nucleosidase, XDH, uricase and allantoinase were also present mainly in cytosol fraction. Hanks *et al.* (1981) while investigating the intracellular localisation of enzymes of ureide biosynthesis in soybean nodules, confirmed the presence of uricase in peroxisomes and that of allantoinase in microsomal fraction. Similarly, in a recent study, Boland *et al.* (1982) indicated the presence of enzymes of purine biosynthesis mainly in proplastids indicating that the complete pathway for purine biosynthesis is located in proplastids. This was confirmed when purine biosynthesis *in vitro* by a proplastid fraction from soybean (Boland and Schubert, 1983) and Cowpea (Shelp *et al*., 1983) nodules was demonstrated. Putting all this information together, a complete pathway of ureide biogenesis from the products of nitrogen fixation in pigeonpea nodules could be visualised as depicted in figure 3. According to this scheme, ammonium is assimilated to the level of glutamine in cytosol. This along with aspartate and other intermediates are utilised for the biosynthesis of purines in proplastids. The end products of purine biosynthesis are oxidised to the level of uric acid in cytosol. Two of the last steps may be occurring in peroxisomes and endoplasmic reticulum, respectively.

References

Atkins, C. A., Pate, J. S., Griffiths, G. J. and White, S. T. (1980a) *Plant Physiol.*, **66,** 978.

Atkins, C. A., Rainbird, R. M. and Pate, J. S. (1980b) *Z. Pflanzenphysiol*., **97,** 249.

Bergmeyer, H. U. and Bernt, E. (1963) in *Methods of Enzymatic analysis*, ed. H. U. Bergmeyer (New York: Academic Press) p. 837.

Boland, M. J. and Schubert, K. R. (1982) *Arch. Biochem. Biophys.*, **213,** 486.

Boland, M. J. and Schubert, K. R. (1983) *Arch. Biochem. Biophys.*, **220,** 179.
Boland, M. J., Fordyce, A. M. and Greenwood, R. M (1978) *Aust. J. Plant Physiol*., **5,** 553.
Boland, M.J., Hanks, J. F., Reynolds, P. H. S., Blevins, D. G., Tolbert, N. E. and Schubert, K. R. (1982) *Planta*, **155,** 45.
Christensen, T, M. I. E. and Jochimsen, B. U. (1983) *Plant Physiol*., **72,** 56.
Fujihara, S. and Yamaguchi, M. (1978) *Plant Physiol*., **62,** 134.
Hanks, J. F., Tolbert, N. E. and Schubert, K. R. (1981) *Plant Physiol.*, **68,** 65.
Hatch, M. D. (1972) *Anal. Biochem.*, **47,** 174.
Herridge, D. E., Atkins, C. A., Pate, J. S. and Rainbird, R. M. (1978) *Plant Physiol*., **62,** 495.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem*., **193,** 265.
Luthra, Y. P., Sheoran, I. S. and Singh, R. (1981) *Curr. Sci*., **50,** 270.
Luthra, Y. P., Sheoran, I. S., Rao, A. S. and Singh, R. (1983a) *J. Exp. Bot.* **34,** 1358.
Luthra, Y. P., Sheoran, I. S., Rao, A. S. and Singh, R. (1983b) *Curr. Sci*., **52**,957.
McClure, P. and Israel, D. W. (1979) *Plant Physiol*., **64,** 411.
Ozbun, J. L, Hawker, J. S., Greenberg, E., Lammel, C. and Preiss, J. (1973) *Plant Physiol*, **51,** 1.
Pate, J. S., Atkins, C. A., White, S. T., Rainbird, R. M. and Woo, K. C. (1978) *Plant Physiol*, **65,** 961.
Reynolds, P. H. S., Boland, M.J., Blevins, D. G., Randall, D. D. and Schubert, K. R. (1982a) *Plant Physiol.*, **69,** 1334.
Reynolds, P. H. S., Blevins, D. J., Boland, M. J., Randall, D. D. and Schubert, K. R. (1982b) *Physiol. Plant*., **55,** 255.
Rogenes, S. E. (1975) *Phytochemistry*, **14,** 1975.
Schubert, K. R. (1981) *Plant Physiol.*, **68,** 1115.
Shelp, B. J. and Atkins, C. A. (1983) *Plant Physiol*., **72,** 1029.
Shelp, B. J., Atkins, C. A., Storer, P. J. and Canvin, D. T. (1983) *Arch. Biochem. Biophys*., **224,** 429.
Sheoran, I. S, Luthra, Y. P., Kuhad, M. S. and Singh, R. (1981) *Phytochemistry*, **20,** 2675.
Sheoran, I. S., Luthra, Y. P., Kuhad, M. S. and Singh, R. (1982) *Plant Physiol.,* **70,** 917.
Streeter, J. G. (1979) *Plant Physiol*., **63,** 478.
Tajima, S. and Yamaraoto, Y. (1975) *Plant Cell Physiol*., **16,** 271.
Tajima, S., Yatazawa, M. and Yamamoto, Y. (1977) *Soil Sci. Plant Nutr.*, **23,** 225.
Triplett, E. W., Blevins, D. G. and Randall, D. D. (1980) *Plant Physiol*., **65,** 1203.
Triplett, E. W., Blevins, D. G. and Randall, D. D. (1982) *Arch. Biochem. Biophys*., **219,** 39.
Wilson, D. O. and Reisenaur, H. M. (1963) *Plant Soil*, **18,** 364.
Wong, P. P. and Evans, H. J. (1971) Plant *Physiol*., **47,** 750.
Woo, K. C., Atkins, C. A. and Pate, J. S. (1980) *Plant Physiol*., **66,** 735.
Woo, K. C., Atkins, C. A. and Pate, J. S. (1981) *Plant Physiol.,* **67,** 1156.