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Ureide Metabolism in Non-nodulated *Phaseolus vulgaris* L.

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ABSTRACT

The distribution of ureide-N was studied throughout vegetative and reproductive growth of non-nodulated *Phaseolus vulgaris* L. (bushbean) grown in nitrate nutrient solution. Largest increases in ureide-N per plant were correlated with flowering and early pod formation and with seed filling. Highest amounts of ureides per organ were measured in stems and axillary trifoliates. Highest concentrations (μmol ureide-N g^{-1} fr. wt.) were measured in young developing organs and stems. Seeds did not accumulate ureides until the ureide content of pods had reached a maximum.

Results obtained using the inhibitor of xanthine oxidase, allopurinol, are consistent with the origin of ureides via purine degradation but alternative pathways cannot be discounted.

Leaves and stems were shown to have the ability to degrade allantoate via an enzymic process.

INTRODUCTION

The occurrence in leguminous and other plants of large amounts of nitrogen in the form of the ureides, allantoin, and allantoic acid (allantoate), was already established by the 1930's by Fosse and coworkers (Tracey, 1955 and references therein). The physiology of these compounds was largely neglected until the work of Mothes and coworkers (Mothes, 1961; Reinbothe and Mothes, 1962) who showed that ureides often accumulated under conditions of carbon starvation and were produced in roots in response to ammonia detoxification (Mothes, 1961). In plants such as comfrey (*Symphytum officinale* L.), ureides ascended in the xylem sap in spring supplying growing regions with nitrogen for amino acid and protein synthesis and in autumn the reverse occurred, with ureides accumulating in storage organs (Mothes and Engelbrecht, 1954).

Early evidence suggested that ureides were produced via purine degradation but a more direct synthesis from urea could not be discounted (Reinbothe and Mothes, 1962). Work on the enzymes involved in purine degradation has been mainly studied in animals and micro-organisms with little emphasis on the occurrence, properties, and location of these enzymes in plants (Vogels and van der Drift, 1976). In particular there have been only a few reports of allantoic acid-degrading activity in plants (Echevin and Brunel, 1937; van der Drift and Vogels, 1966;

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Singh, 1968; Hartmann and Arnold, 1974; Tajima, Yatazawa, and Yamamoto, 1977). Following the work of Kushizaki, Ishizuka, and Akamatsu (1964) and Ishizuka, Okino, and Hoshi (1970) who showed that nodulated soyabean plants (*Glycine max* L.) contain large amounts of ureides, two Japanese groups have published evidence that nodules produce allantoin (Matsumoto, Yatazawa, and Yamamoto, 1977*a*; Fujihara and Yamaguchi, 1978*a*). From measurements of the enzymes involved in purine catabolism it was suggested that the allantoin is utilized mainly in the leaves after translocation (Tajima *et al.*, 1977).

Although reports on the occurrence and distribution of ureides in leguminous plants have been mainly on nodulated plants, significant amounts have been reported in non-nodulated soyabeans (Matsumoto *et al.*, 1977*b*) and young bushbeans (*Phaseolus vulgaris* L.) (Engelbrecht, 1955). We have reported elsewhere that allantoic acid is an important N transport compound throughout the life cycle of bushbeans (Thomas, Feller, and Erismann, 1979) and in this paper we describe the occurrence and distribution of ureides and some aspects of ureide formation and degradation in non-nodulated bean plants.

MATERIALS AND METHODS

Plant material and growth

Seeds of *Phaseolus vulgaris* L. cv. Saxa (Radio) Stamm Vatter (bushbean) were germinated and grown in liquid cultures as described previously (Thomas *et al.*, 1979). The source of nitrogen for nitrate-grown plants was 0·317 g $Ca(NO_3)_2.4H_2O$ and 0·088 g KNO_3 per litre (total N 3·5 mM) and 0·236 g $(NH_4)_2SO_4$ was used for ammonium nutrient solutions (total N 3·5 mM).

Collection of xylem and phloem sap

Xylem bleeding sap was collected as before (Thomas *et al.*, 1979) and the method used to obtain phloem sap was basically that of King and Zeevaart (1974). Petioles were cut obliquely with a sharp razor blade under solution and placed in 10 ml 20 mM Na_2EDTA for 2 h in darkness. The resulting solution contained high levels of sugars and only traces of nitrate, indicating little contamination from either xylem sap or liquid from damaged tissues.

Preparation of tissue extracts for the estimation of ureides

To approximately 1 g fr. wt. finely chopped tissue was added 4–5 ml distilled water and extracts were prepared by homogenization at speed 7 in a Polytron homogenizer (Kinematica, Luzern) for 45–90 s. The temperature of the extracts varied between 30 and 40 °C. Extracts were passed through four layers of Miracloth (Calbiochem, San Diego) and filtrates were centrifuged for 20 min at 3000 *g* in a cold centrifuge (0 °C) and either analysed immediately or stored at −20 °C. For each root extract equal weights were cut from the tip, middle, and basal root parts. Portions of stem from each node and internode were sampled for stem extracts and all leaf extracts included petioles. Fruits were separated into pods and seeds in plants harvested after 30 d growth and extracts of dry tissues were made with boiling water. Material from six plants was used for extracts of 7–14 d old plants and from at least two plants for extracts of older plants.

Estimation of ureides and TCA-soluble N in plant extracts

Ureides and their derivatives were determined by a differential analysis of glyoxylate derivatives (Vogels and van der Drift, 1970). To 0·5 ml water extract was added 0·5 ml cold 10% (w/v) TCA and samples were incubated in iced water (0 °C) for 15 min. The resulting white precipitates were removed by centrifugation and TCA-soluble N was determined in the supernatants using a micro-Kjeldahl procedure (Bohley, 1967).

Measurement of enzyme activities

Enzyme extracts were prepared as described previously (Thomas *et al.*, 1979) using cold 0·1 M phosphate buffer, pH 7·5, containing 1 mM Na_2EDTA, 1 mM dithioerithritol, and 1% (w/v) casein.

For the assay of allantoicase activity the cheesecloth filtrate was centrifuged at 3000 *g* for 10 min in a cold (0 °C) centrifuge. The resulting supernatant was passed over a Sephadex G-25 column by centrifugation to remove low molecular weight compounds (Feller, Soong, and Hageman, 1977). The cheesecloth filtrate was used directly in the assay of nitrate reductase and glutamine synthetase, Sephadex G-25 treatment having no effect on enzyme activities in preliminary experiments.

For the allantoicase assay each tube contained 50 μmol phosphate buffer, pH 7·5, 15 μmol sodium allantoate, and 2–3 drops 0·33% (w/v) phenylhydrazine–HCl adjusted with H_2O to 0·4 ml. The reaction was started by the addition of 0·1 ml desalted enzyme extract giving a final volume of 0·5 ml and incubation was for 15 min at 30 °C. The assay tubes were then placed in ice water and 0·25 ml incubation mixture was removed and analysed immediately for the production of ureidoglycolate; the remaining 0·25 ml was used for the determination of glyoxylate (Vogels and van der Drift, 1970). The optical densities of two controls, one minus extract (measuring non-enzymic breakdown), the other minus substrate (measuring free glyoxylate), were substracted from experimental readings. Activities are expressed in μmol allantoate broken down h^{-1} g^{-1} fr. wt.

Nitrate reductase activity was measured in root and leaf extracts as described earlier (Thomas *et al.*, 1979).

The incubation mixture for glutamine synthetase contained, in a final volume of 1·0 ml: 112 μmol $MgSO_4$, 230 μmol L-glutamate, 12 μmol NH_2OH, 36 μmol ATP, and 125 μmol imidazole. The pH of a stock solution with $MgSO_4$, glutamate, and imidazole was adjusted to 7·2. NH_2OH and ATP solutions were prepared freshly before each experiment. The reaction was started by the addition of ATP (0·1 ml 360 mM ATP neutralized to pH 7·0). After 15 min incubation at 30 °C the reaction was stopped by the addition of 1 ml $FeCl_3$ solution (O'Neal and Joy, 1973). Glutamyl hydroxamate was used as the standard and activities are expressed in μmol hydroxamate produced h^{-1} g^{-1} fr. wt.

Inhibitor experiments

Nitrate-grown plants were placed in plastic beakers containing 500 or 750 ml ammonium nutrient solution to stimulate ureide production in roots (three plants per beaker, two beakers, per treatment) and were fed with and without allopurinol (4-hydroxypyrazolo-(3,4-d)pyrimidine; Sigma Chemical Co.), an inhibitor of xanthine oxidase (Fujihara and Yamaguchi, 1978*b*), at a final concentration of 0·5 mM and aerated for varying periods of time. At the end of the feeding period xylem sap was collected from the stem bases for not more than 1 h and analysed for nitrate, Kjeldahl-N, and allantoate-N (Thomas *et al.*, 1979).

RESULTS

Effect of allopurinol on the presence of allantoate in xylem sap

The production of ureides in roots is thought to be mainly via purine degradation (Mothes, 1961) and soyabeans treated with allopurinol, an inhibitor of xanthine oxidase, contained low amounts of ureides and accumulated xanthine compared with controls (Fujihara and Yamaguchi, 1978*b*).

Addition of 0·5 mM allopurinol to the medium of 16 d old nitrate-grown plants had no inhibitory effect on nitrate reductase activity in either root or leaf extracts (102 and 104% respectively of an appropriate control without allopurinol). Glutamine synthetase activity was slightly higher in roots (165%) and was not changed in leaves (99%) after a 5 h feeding period. The effects of feeding whole plants via the roots with allopurinol on xylem sap N are shown in Table 1. In experiment I 12 d old nitrate-grown plants were transferred to ammonium nutrient solution to stimulate the production of allantoate (Thomas *et al.*, 1979) and incubated with 0·5 mM allopurinol. After 68 h xylem sap was collected from the base of the stems and analysed. The concentrations of Kjeldahl-N and total N were similar while nitrate was higher in sap collected from allopurinol-treated plants compared with controls, but the concentration of allantoate was decreased by 92% in plants given the inhibitor. A similar result was obtained when the experiment was

repeated, collecting sap after 5 h feeding (experiment II). Allantoate was 50% less in the sap of allopurinol-treated plants while Kjeldahl-N and nitrate were more or less unchanged compared with controls. In experiment III plants were fed with arginine, a source of N likely to promote urea production within the plant via arginase (Jones and Boulter, 1968) and the effects of arginine and allopurinol feeding on xylem sap N are shown in Table 1. After 4 h feeding with allopurinol the concentration of allantoate in the xylem sap was 57% less than that in controls given no inhibitor and, in plants given arginine plus allopurinol, allantoate was decreased by 48% even though Kjeldahl-N was increased by 125% compared with the control (no addition). Thus, while arginine appeared to be taken up by the

TABLE 1. *Effect of allopurinol on xylem sap N*

Nitrate-grown plants 12 d old were transferred to ammonium nutrient solution with and without 0·5 mM allopurinol. After 68 h (Expt. I) and 5 h (Expt. II) xylem sap was collected from six plants per treatment and analysed for N. In Expt. III 15 d old plants were transferred to ammonium nutrient solution for 6 d and six plants per treatment were given fresh ammonium solution, ammonium solution plus 0·5 mM allopurinol, or ammonium solution plus 0·5 mM allopurinol plus 50 mM L-arginine. Xylem sap was collected after 4 h feeding.

	Treatment	Kjeldahl-N	Nitrate-N	Allantoate-N	Total N (Kjeldahl-N + Nitrate-N)
Expt. I	Control	60·71	3·28	9·80	63·99
	+ Allopurinol	57·57	6·71	0·82	64·28
Expt. II	Control	30·71	11·14	6·80	41·85
	+ Allopurinol	27·14	11·18	3·40	39·32
Expt. III	Control	17·14	14·28	4·40	31·42
	+ Allopurinol	15·36	13·57	1·90	28·93
	+ Allopurinol + arginine	38·36	10·00	2·30	48·86

plants and either transported directly or assimilated and then transported to the xylem, there was no evidence of a stimulation of allantoate production via a direct urea-based synthesis.

We failed to obtain evidence that allantoate might be synthesized via a urea-based metabolism (e.g. a condensation of glyoxylate and urea: Reinbothe and Mothes, 1962; Atkins, Herridge, and Pate, 1978). Incubation of root extracts with urea and glyoxylate using a range of Tris–HCl buffers, pH 8·0–8·6, did not result in the production of allantoate (results not included). However this result cannot rule out the urea-based synthesis *in vivo*.

Allantoicase activity in leaves and stems

In vitro activities of allantoicase were measured in extracts of 10 d old nitrate-grown plants. Stem extracts hydrolysed 8·27 and those from primary leaves 8·04 μmol allantoate h^{-1} g^{-1} fr. wt. In roots little allantoicase activity could be detected.

Comparison of total ureide-N in nitrate- and ammonium nitrate-grown plants

Bleeding sap of ammonium nitrate grown plants contains considerably more allantoate-N than nitrate-grown plants (Thomas *et al.*, 1979) and Engelbrecht (1955) reported that allantoin was present in greater quantities than allantoic acid in plants grown in soil enriched with ammonium nitrate. Table 2 compares the distribution of ureides in 14 d old nitrate- and ammonium nitrate-grown plants. Greatest amounts of ureides were measured in the stems and primary leaves of plants grown with either N source, and highest concentrations (μmol g^{-1} fr. wt.) were measured in the very young second trifoliate leaves and growing tip. Ammonium nitrate-grown plants contained 45% more total ureide-N than nitrate-grown plants with 66% more allantoin and 38% more allantoic acid. In general, in each plant organ, allantoic acid was the predominant form of ureide regardless of the N source contributing about 75% to the total ureide-N.

TABLE 2. *Comparison of the distribution of ureide-N in 14 d old nitrate- and ammonium nitrate-grown plants*

Ureides were measured in warm water extracts. Figures in brackets are the amounts of allantoate expressed as % total ureide-N.

Organ	μmol ureide-N g^{-1} fr. wt. or per organ			
	Nitrate-grown		Ammonium nitrate-grown	
	g^{-1} fr. wt.	per organ	g^{-1} fr. wt.	per organ
Root	1·22	1·94 (93)	1·27	1·85 (100)
Lower stem (up to cotyledons)	6·75	2·73 (69)	10·09	3·91 (78)
Upper stem (above cotyledons)	6·90	2·79 (76)	14·41	5·57 (80)
Primary leaf	2·47	6·25 (73)	3·78	8·72 (59)
1st trifoliate	6·64	1·01 (84)	8·79	1·49 (82)
2nd trifoliate + growing tip	18·64	0·59 (75)	18·44	0·64 (85)
Total ureide-N per plant	—	15·31 (77)	—	22·18 (74)
Total allantoate-N per plant	—	11·78	—	16·41

Distribution of ureides in nitrate grown plants

The amounts and concentrations of ureides measured in the different organs of nitrate-grown plants throughout vegetative and reproductive growth are shown in Fig. 1. Cotyledons were analysed from the first day after transfer to liquid cultures (day 7) and contained low amounts of ureides compared with other organs. There was a rapid decrease in ureide content as the cotyledons aged. By day 14 most cotyledons had fallen off the plant.

In roots there was a gradual increase in the ureide content up to day 51 when an apparent plateau was noted which was followed by two increases which were larger than that measured prior to day 51 (days 58 and 72). The concentration of ureides in roots was initially high (day 7) and then decreased and remained approximately

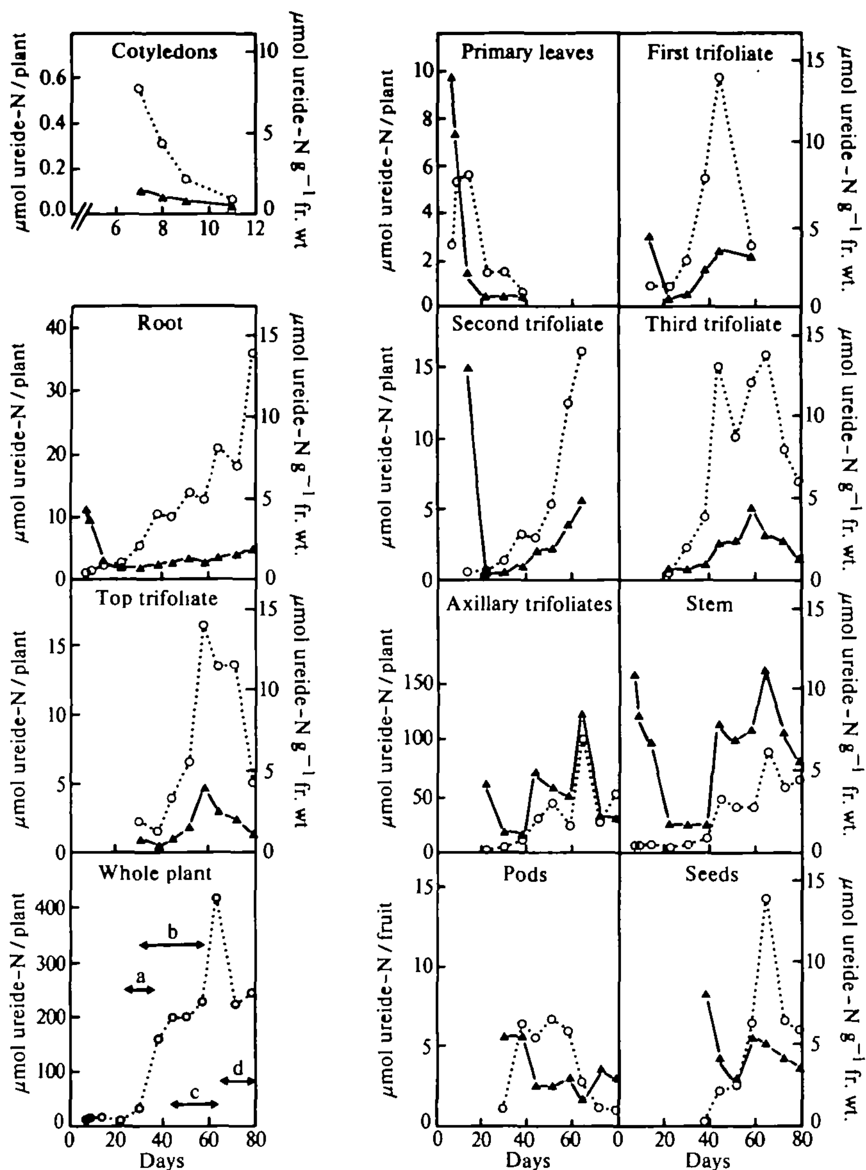

FIG. 1. Concentrations and contents of ureides in different parts of nitrate-grown plants. Ureides (allantoin + allantoic acid) were measured in tissue extracts and results are means of two replicate samples from two or three separate experiments. All pods were assumed to contain five seeds (the mean) for the calculation of total ureide-N in fruits. Each point in the 'whole plant' diagram is the sum total of ureide-N in all organs still attached to the plant at the time of harvest. The letters a (flowering), b (pod formation), c (seed filling), and d (pod desiccation) refer to stages of reproductive growth. Key: O· · · ·O, ureide-N contents (left scale); ▲——▲, ureide-N concentrations in μmol N g^{-1} fr. wt. (right scale).

constant over the development of the plant contributing an average of 4% to the TCA-soluble N in root extracts.

The amounts of ureides measured in stems showed several phases. Initially the total ureide-N remained constant before decreasing after 14 d growth. This was

followed by an increase which stopped after day 44 and then sharply increased after day 58 before finally decreasing between days 64 and 72. The concentration of ureides followed a similar pattern (Fig. 1) and on average made up 9·5% of the TCA-soluble N which was the highest mean concentration of all organs studied during the development of the plants.

The pattern of the ureide content in primary, first, second, third, and top trifoliate leaves was essentially the same in each leaf. Young leaves contained low amounts of ureides but the concentration was often high, particularly in primary leaves. As the leaves developed the ureide content increased gradually, reaching a maximum when senescence was already evident (yellowing leaves). Thereafter the ureide content decreased and in leaves recently fallen from the plant only low amounts were detected. Furthermore samples of phloem sap collected from senescing leaves contained low amounts of ureides (results not shown) indicating that N in the form of ureides was re-translocated out of the older leaves.

The total amount of ureides in all axillary trifoliates (i.e. trifoliates arising from axillary buds) followed a similar pattern to that described for stems except that there was no initial decrease in ureide content. Instead ureides increased rapidly up to day 44, then levelled off before increasing rapidly once more after approximately 58 d growth. The total amounts in the axillary trifoliates were of the same order as those measured in stems.

Figure 1 shows that the major increases in ureide content per plant were correlated with developmental changes associated with reproductive growth and fruit formation. At the onset of flowering there was a rapid increase in the amount of ureides within the plant which continued until early pod formation. The increase in ureides then stopped until seed filling became the predominating process (at approximately 58 d growth) when there were further large increases in the amounts of ureides in roots, stems, and axillary trifoliates (Fig. 1) followed by a decrease when the pods had begun to desiccate. These correlations were noted in three separate experiments (fruits were analysed in two of these experiments) in which plants were grown at different times.

The distribution of ureides in fruits is also shown in Fig. 1. For these measurements the first pods to develop were chosen (at the top of the plant) as representative of all the pods on the plant. Initially pods contained most of the ureides and seeds did not begin to accumulate ureides until the amounts in the pods had reached a maximum (days 40 to 51). After day 51 the ureide content of the seeds increased more rapidly and reached a maximum on day 64 and then decreased. The ureide contents of pods began to decrease after 58 d growth at a time when the accumulation in seeds was most rapid.

DISCUSSION

The major increases in the amounts of ureides in bean plants were correlated with two phases of reproductive growth, namely flowering and early pod formation and seed filling. From our earlier findings (Thomas *et al.*, 1979) that N transport in the xylem reached a maximum at flowering and that there was an increase in the

concentration of xylem sap N after 8 weeks growth (seed filling) we conclude that the observed increases in ureides within the plant are associated with increases in N transport from the root via the xylem. These observations taken together with the findings (1) that highest concentrations of ureides were measured in stems, young developing organs, pods, and seeds, and (2) that although ureides can contribute 13–42% to the total xylem sap N (Thomas *et al.*, 1979) generally they contributed not more than and often much less than 10% to the TCA-soluble N in different organs, indicate that ureides are important in both storage and translocation of N as previously suggested (Mothes, 1961).

In an earlier study Engelbrecht (1955) found that the ureide content of young *P. vulgaris* plants decreased after 31 d growth. However when the development of the plants in her and our studies are compared (by comparison of the ratios of the fresh weights of primary leaves and trifoliate leaves) 16 d growth in our study corresponds to 31 d growth in the former study and we also observed a decrease in ureide content per plant at this stage of development. The most likely explanation for the decrease is that it occurs at a time when plants have utilized all the reserves from the cotyledons but before the leaves have started to export carbon assimilates to other plant parts such as the roots which consequently have a lowered assimilatory capacity. Engelbrecht, who grew her plants on soil enriched with ammonium nitrate, also reported in the same study that allantoin was the major form of ureide while in our study, with nitrate-grown plants, allantoic acid (allantoate) was always the predominant form. The difference in N source can only partly explain this difference as, although ammonium nitrate grown plants contained 66% more allantoin than nitrate-grown plants, allantoate was still the predominant form. Possibly the different growth conditions and differences in extraction methods are responsible for the conflicting results.

As reported for total N (Oliker, Poljakoff-Mayber, and Mayer, 1978) ureides only began to accumulate in seeds when they had reached a maximum in pods, indicating that both pods and seeds may act as separate and possibly competing sinks.

From the experiments with allopurinol, an inhibitor of xanthine oxidase, and the lack of evidence for a direct synthesis of allantoate from urea and glyoxylate (see schemes of Atkins *et al.*, 1978) our results are consistent with the synthesis of the ureides via purine breakdown but other pathways cannot be completely ruled out. The exact pathway of reduced N into allantoic acid has yet to be confirmed although evidence from nodulated cowpea (*Vigna unguiculata* L.) indicates that both recently fixed C and N (from N_2 gas) are involved (Herridge, Atkins, Pate, and Rainbird, 1978).

Leaves and stems were shown to have the ability to degrade allantoate in a manner similar to that of micro-organisms (Vogels and van der Drift, 1976). There is no information on how this process is regulated in higher plants.

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