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The Assimilation of Ureides in Shoot Tissues of Soybeans[1]

1. CHANGES IN ALLANTOINASE ACTIVITY AND UREIDE CONTENTS OF LEAVES AND FRUITS

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ABSTRACT

The ureides, allantoin and allantoic acid, are major forms of N transported from nodules to shoots in soybeans (Merr.). Little is known about the occurrence, localization, or properties of the enzymes involved in the assimilation of ureides in shoot tissues. We have examined the capacity of the shoot tissues to assimilate allantoin via allantoinase (EC 3.5.2.5) during leaf and fruit development in nodulated soybeans. Specific activity of allantoinase in leaves peaked during pod formation and early seed filling. In developing fruits allantoinase activity in the seeds was 2 to 4 times that in the pods when expressed on a fresh weight or organ basis. In seeds, the embryos contained the highest specific allantoinase activity. Stems and petioles also had appreciable allantoinase activity. With development, peaks in the amounts of allantoic acid, but not allantoin, were measured in both leaves and fruits suggesting that the assimilation of allantoic acid may be a limiting factor in ureide assimilation. Highest amounts of ureides were measured in the pith and xylem of stem tissues and in developing pod walls.

The ureides, allantoin and allantoic acid, are found in abundance in soybeans (5, 8, 10–12). In plants depending solely on N_2 fixation for their N requirements, ureides comprise up to 86% of the xylem sap N (9). Based on enzymic studies, ureides were suggested to be synthesized in nodules and transported to the shoot where they are assimilated (18, 19). Ishizuka (7) has suggested that ureide-N, arising predominantly from N_2 fixation, is used more efficiently in seed protein production than N in the form of amino acids, amides and nitrate. The latter are the major forms of nitrogen exported from the roots when nitrate fertilizers are fed to plants (9).

While ureides are known to accumulate in fruits and are thought to be utilized in seed protein production (7, 12), the enzymes involved in ureide assimilation (allantoinase, allantoicase and urease) have not been previously studied in soybean fruits. We know little about the assimilation of xylem-borne ureides in shoot tissue and the purpose of this paper is primarily to assess the capacity of shoot tissue to assimilate allantoin via allantoinase and to describe the changes in enzyme activity during leaf and fruit development in nodulated soybeans. The amounts of ureides in plant tissues were also examined. The role of stem and petiole tissue in ureide assimilation is discussed.

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MATERIALS AND METHODS

Growth of Plants. Soybean (*Glycine max* [L.] Merr. cv. Wells) seeds were imbibed in aerated distilled H_2O for 4 to 6 h and placed in 20-cm diameter pots containing a soil/sand mixture (50:50). The mixture was steam sterilized prior to use. Seeds were inoculated with approximately 0.2 g *Rhizobium japonicum* (Nitragen Co., Milwaukee, WI). Pots were kept well watered with nutrient solution (2) containing 2 mM KNO_3 for the first 2 days' growth and thereafter with daily and alternating waterings with water or nutrient solution minus N. Plants were grown in growth chambers with a 16-h photoperiod and day/night temperatures of 28 to 30 and 22 to 23 C, respectively. Illumination was provided by fluorescent (92% input wattage) and incandescent (8% input wattage) lighting, giving a photosynthetic photon flux density of 300 ± 25 $\mu E\ m^{-2}\ s^{-1}$ at the surface of the pots. RH was 45 to 50% during the light cycle.

Preparation of Plant Extracts. Extracts for the measurement of enzyme activity and ureide content were prepared by adding 7 ml ice-cold 0.05 M Tris-HCl buffer (pH 7.4) to 1 g fresh weight tissue and homogenizing in a Virtis 60 K homogenizer, speed setting 70, for 1 min. The homogenates were squeezed through four layers of cheesecloth and the filtrate centrifuged at 50,000*g* for 30 min at 0 C. The resulting supernatants were used for the measurement of ureides; for measurements of enzyme activity the extracts were first desalted by passage through a Sephadex G-25 column (3). Extracts of leaves were prepared after removing the petioles and midrib veins, and when possible, fruits were separated into pods and seeds. Fruits developing at the eleventh to thirteenth nodes were used for enzyme extractions. The stem was cut into three sections: (*a*) from nodes 2 to 5; (*b*) nodes 6 to 9; and (*c*) nodes 10 to 14. The tissue from each section was separated into an outer green part (containing cortex, phloem and cambium) and an inner nongreen part (containing pith and xylem) by slicing the outer tissue with a razor blade and peeling it away from the inner tissue. Seeds were separated into seed coats, cotyledons, and embryos. After storage in a freezer (−20 C), seed coats and cotyledons were milled in a cyclone sample mill (Udy Analyzer Co., Boulder, CO). Embryos were not milled. Extracts of the separated seed parts were then prepared as described above.

Measurement of Allantoinase Activity. The method used was basically that of Van Der Drift and Vogels (22) except that a substrate concentration of 25 mM was used. After 15 min incubation at 30 C the reaction was stopped by placing the tubes in ice-cold water and adding 1 drop of concentrated HCl. A 250-μl aliquot was removed from the reaction mixture for the determination of the allantoic acid formed (26).

Absorbance readings were corrected for nonenzymic degradation of allantoin and for any glyoxylate derivatives present in boiled extracts. Using the assay conditions described above, we could detect no allantoicase activity which may have interfered in the assay of allantoinase. In some experiments enzyme activity was also assayed in the pellets resulting from centrifugation at

50,000*g*. Pellets were washed once and resuspended in extraction buffer and sonicated for 30 s with an ultrasonic probe (Blackstone Ultrasonics Inc., Sheffield PA). The resulting extract was used for the assay. Production of allantoic acid was linear with time up to 30 min and with 0.05 to 0.15 ml enzyme extract. Protein was measured in extracts after the method of Bradford (1).

Estimation of Ureides in Plant Extracts. To 1 ml tissue extract was added 0.14 ml 40% (w/v) trichloroacetic acid (final concentration 5%). Tubes were incubated in ice-cold water for 10 min and the resulting precipitate was removed by centrifugation at 12,000*g* for 15 min. Sample volumes up to 0.25 ml were used for the determination of ureides (26). Extraction of ureides with Tris buffer followed by trichloroacetic precipitation was preferred over extraction with 75% ethanol (17) because (a) greater amounts of total ureide (up to 20% more) and allantoic acid were extracted from fruits using Tris and trichloroacetic and (b) ethanolic extracts of leaves and pods frequently contained a cloudy suspension which interfered in the colorimetric assay for glyoxylate derivatives (26).

RESULTS

Changes in Allantoinase Activity and Ureide Concentration During the Development of a Trifoliolate Leaf. Specific activity of allantoinase, measured in the supernatants of extracts from the lamina during the development of the eighth trifoliolate leaf, was high in the young unexpanded leaf but decreased during leaf expansion (as measured by the increase in length of the terminal leaflet) (Fig. 1). The activity then increased reaching a peak during pod formation and early seed filling in fruits developing in the axils of the eighth trifoliolate leaf. The activity declined during seed filling before increasing again during leaf senescence. On a per leaf basis, allantoinase activity peaked a few days after the initiation of flowering on day 49. Thereafter, the enzyme activity decreased markedly before finally increasing slightly during leaf senescence. On the average, 54% of the total extractable activity was measured in the washed 50,000*g* pellet. Inclusion of the activity measured in the pellet with that measured in the supernatants did not markedly alter the pattern of activity during leaf development.

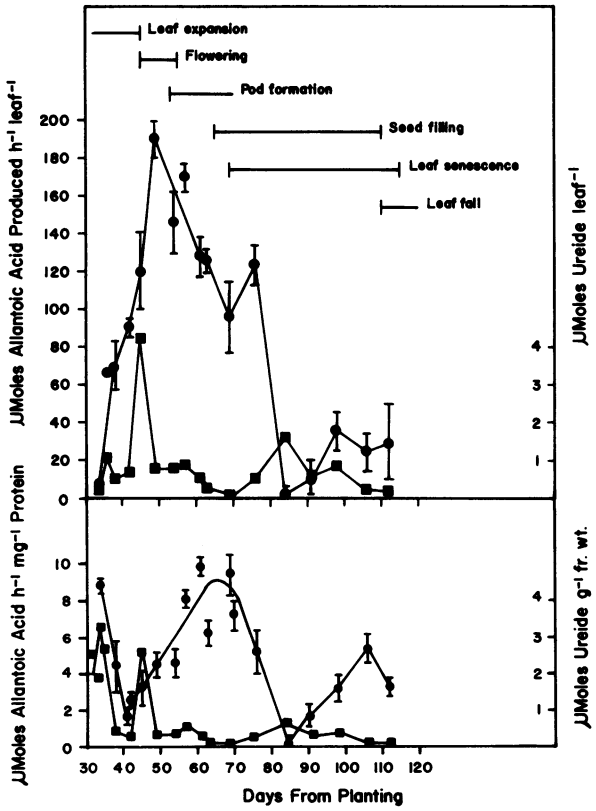

FIG. 1. Changes in allantoinase activity during the development of the eighth trifoliolate leaf. Allantoinase activity and ureides were measured in 50,000*g* supernatants of leaf lamina extracts as described. ●——●, allantoinase activity; ■——■, total ureides (allantoin + allantoic acid). Results are means of four replicate samples. Bars represent SE. Values for ureide content and concentration per leaf ± SE were generally no larger than the symbols and consequently error bars were not included.

The ureide concentration in the trifoliolate leaf was high in young unexpanded leaves and decreased during leaf expansion (Fig. 1). A sharp increase in ureide concentration and content occurred at day 45 (flowering). At this time 80% of the total ureide was in the form of allantoic acid. Following this peak the ureide concentration remained low during fruit development, rising slightly only when senescence of the leaf was evident (yellowing). Apart from the noted peak, 57% of the total ureide was in the form of allantoic acid, the remainder being allantoin.

Allantoinase Activity and Ureide Content of Stem, Leaf, and Petiole. Table 1 shows that in the stem there was a gradient of decreasing allantoinase activity from the lower to the upper nodes. Greater activity was measured in the outer tissues (cortex, phloem, and cambium) compared with the inner tissues (pith and xylem). No activity could be detected in the uppermost nodes even though ureides were measured in extracts of these nodes. The lower nodes contained the lowest concentrations of ureides; the middle and upper nodes contained similar concentrations. This distribution pattern of ureides in stem tissues is similar to that reported earlier (12). However, Matsumoto *et al.* (12) did not separate the stem into inner and outer tissues. We observed that the inner stem tissues contained greater amounts of ureides on an organ basis compared with the outer tissues although concentrations were similar (Table 1).

During leaf development the specific activity in the lamina increased whereas that in the petiole decreased (Table 1). The concentration and content of ureides were highest in both lamina and petiole on day 45 (flowering).

Changes in Allantoinase Activity and Ureide Concentrations in Developing Fruits. Figure 2 shows the sum of the allantoinase activities measured in 50,000*g* supernatants and pellets during fruit development. Allantoinase activity (per g fresh weight) of pods was highest at the initial sample, 60 days after planting. Because of the small sample size, fruits were not separated into pods and seeds until after pod elongation. During seed filling, the activity in the pods increased slightly and remained at a relatively constant level of about 150 μmol allantoic acid produced per h per g fresh weight up until 110 days' growth. The activity thereafter decreased, the beginning of the decrease occurring simultaneously with the beginning of pod senescence (yellowing). The enzyme activity in the seeds increased markedly with early seed filling, reaching levels nearly five times that measured in the pods at 86 days' growth. This peak in activity was followed by a rapid decrease between days 86 and 97 which became more gradual during the remainder of seed development. The allantoinase activity in the seeds remained about three times that in the pods during the entire period of seed filling. During pod and seed desiccation (after 120 days' growth) the activity in the pods was minimal whereas that in the seeds was still appreciable. On the average the 50,000*g* pellets of both pods and seeds contained 58 and 49% of the total allantoinase activity, respectively, and the level decreased to below 25% only during pod and seed desiccation. On an organ basis, the enzyme activity in the seeds increased rapidly during early seed development (days 78–86) and continued to increase, but at a less rapid rate, throughout the rest of the seed filling period (days 86–121). A decrease in activity in the seeds

Table I. *Comparison of Allantoinase Activity and Ureide Content of Stem, Leaf, and Petiole*

Allantoinase activity and ureide contents were measured in tissue extracts prepared as described. Enzyme activities are sums of activities measured in 50,000*g* supernatants and pellets. Stems were separated into an outer portion (cortex, phloem, and cambium) and an inner portion (pith and xylem). Results are means of four replicate samples ± SE.

Plant Age in Days	Tissue	Allantoinase Activity (μmol allantoate produced h^{-1})		Total Ureide Content (μmol)	
		mg^{-1} protein	*g^{-1} fresh wt*	*$organ^{-1}$*	*g^{-1} fresh wt*
	Stem				
42	Nodes 2–5 outer	16.5 ± 1.5	73.4 ± 8.0	0.69 ± 0.06	0.64 ± 0.05
	Nodes inner	11.6 ± 2.9	18.8 ± 4.7	2.70 ± 0.80	0.98 ± 0.28
	Nodes 6–9 outer	11.1 ± 1.2	51.7 ± 6.7	2.74 ± 0.06	3.26 ± 0.06
	Nodes inner	5.6 ± 0.7	11.4 ± 1.4	6.62 ± 0.26	3.47 ± 0.14
	Nodes 10–14 outer	0	0	1.36 ± 0.14	3.23 ± 0.32
	Nodes inner	0	0	4.72 ± 0.28	4.77 ± 0.28
	8th Trifoliolate				
42	Lamina	4.9 ± 0.5	119.7 ± 9.6	0.76 ± 0.37	0.35 ± 0.17
	Petiole	20.4 ± 3.7	99.5 ± 17.9	0.48 ± 0.24	0.69 ± 0.34
45	Lamina	5.27 ± 0.6	173.1 ± 20.8	4.27 ± 0.56	2.63 ± 0.34
	Petiole	18.7 ± 3.4	137.9 ± 24.8	2.66 ± 0.81	6.87 ± 2.06
49	Lamina	6.3 ± 0.6	153.7 ± 23.0	0.81 ± 0.38	0.33 ± 0.15
	Petiole	9.4 ± 4.7	35.9 ± 20.4	0.11 ± 0.07	0.20 ± 0.01

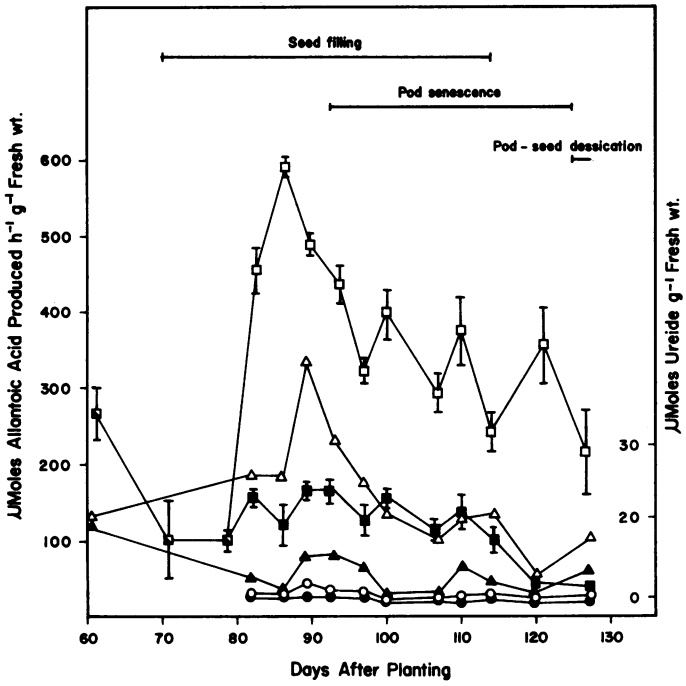

FIG. 2. Allantoinase activity and concentration of ureides in developing fruits. Allantoinase activity and ureides were measured in extracts of fruits developing on the eleventh to thirteenth nodes. Allantoinase activity in seeds, □——□; fruits, ▲——▲; pod walls, ■——■. Total ureide (allantoin + allantoic acid) concentration in pod walls, △——△; and seeds, ○——○. Allantoin concentration in pod walls, ▲——▲; and seeds, ●——●. Enzyme activities are sums of the activities measured in 50,000*g* supernatants and pellets. Results are means of four replicate samples. Bars represent SE. Values for the concentrations of ureides ± SE were generally no larger than the symbols and consequently error bars were not included.

was noted when the seeds had begun to desiccate (results not shown). With the exception of a low activity per fruit between days 60 and 80 the activity per pod followed a similar pattern to that described on a fresh weight basis (Fig. 2).

The total ureide concentration (allantoin plus allantoic acid) of the pods fluctuated during fruit development reaching a peak at approximately 89 days' growth, just before the beginning of pod senescence (Fig. 2). The ureide concentration then decreased steadily during pod senescence. Although total ureide concentration (allantoin plus allantoic acid) changed during fruit development, the concentration of allantoin did not change greatly. These results show that allantoic acid accumulates in pods during early seed filling. Both total ureide and allantoic acid concentrations in seeds were low compared with those in the pods at all stages of fruit development. The fluctuations in the amounts of ureides in pods and seeds during fruit development were similar when results were expressed on either a concentration basis (μmol/g fresh weight) or on a total content basis (μmol/pod or three seeds).

Table II. *Allantoinase Activity and Ureide Concentrations in Seed Parts*

Fruits were harvested from 110-day-old plants (maximum seed fresh wt). Allantoinase activity and ureides were measured in 50,000*g* supernatants. Results are means of four replicates ± SE.

Tissue	μmol Allantoate Produced h^{-1}		Total Ureide Concentration
	g^{-1} fresh wt	*mg^{-1} protein*	*μmol g^{-1} fresh wt*
Seed coat	112.3 ± 27.1	6.75 ± 1.54	2.22 ± 0.03
Cotyledons	549.1 ± 48.4	5.85 ± 0.21	0.54 ± 0.14
Embryo	1,960.0 ± 178.7	21.14 ± 0.10	0.72 ± 0.26

Allantoinase activity and concentration of ureides in different parts of the seed are shown in Table II. The seeds at this stage of development (110 days old) were green and had attained maximum fresh weight. Highest activity per g fresh weight or per mg protein was measured in the embryo axis with three to seventeen times as much activity per g fresh weight compared to that measured in the cotyledons and seed coat. The concentration of ureides was highest in the seed coat, being three to four times that in either the embryo axis or cotyledons.

DISCUSSION

The results show that all parts of the shoot have some capacity to assimilate allantoin to allantoic acid. Previous work (18, 19) has shown that allantoinase is present in stem and leaf tissue, but prior to this report there has been no information on the presence of ureide-assimilating enzymes in soybean fruits. During leaf

development greatest specific activities of allantoinase were measured after leaf expansion and during flower and pod formation. As ureides are the major forms of N in the xylem sap of nodulated plants (13) and assuming that ureides are transported to the leaves in the xylem via mass flow in the transpiration stream, the influx of ureides into the leaves will also be greatest during the period of high enzyme activity. It is unlikely that there is an increase in flux of ureides into the leaves during senescence and drying as transpiration rate will be low. Therefore the increase in specific activity during leaf senescence indicates that this enzyme may be important in the mobilization of N from purines and/or that it is in some way more stable than other proteins to proteolysis.

The only notable change in ureide concentration in leaves was an increase in allantoic acid (up to 80% of the total ureide) at a time when allantoinase activity was increasing (Fig. 1, day 45). This plus the finding that allantoic acid accumulated in pod walls (Fig. 2), suggests that allantoinase may be limiting ureide assimilation. In leaves, generally 57% of the total ureide was in the form of allantoic acid; similarly allantoic acid was the predominant ureide in fruit tissues. This ratio of allantoin:allantoic acid differs from that in an earlier report (17). Streeter reported a ratio of 60:40 for allantoin:allantoic acid in leaf and fruit tissue. Allantoic acid is the predominant form of ureide in the xylem sap (9, 13) and so a higher ratio of allantoin:allantoic acid would imply that allantoic acid is used preferentially over allantoin. The high levels of allantoinase and accumulation of allantoic acid reported here are inconsistent with this idea. Allantoin and allantoic acid are unstable compounds susceptible to degradation under mild conditions of temperature and pH (24, 25). It is possible that the differences in ratio of allantoin:allantoic acid between our and Streeter's work (17) are due to differences in the handling, storage and extraction of the ureides. Comparison of the methods is difficult due to the long storage period involved in the latter work.

In both leaves and fruits, at least 50% of the allantoinase activity was measured in the 50,000g pellet. Attempts to localize the enzyme have so far proved unsuccessful because of difficulties in isolating cell organelles. It seems that a substantial amount of enzyme activity is associated with a membrane and/or organelle. Allantoinase has previously been reported to be associated with microbodies in various plant tissues (15, 16, 20). The distribution of allantoinase activity between the pod and seeds is similar to that reported for cowpeas (*Vigna unguiculata* L.) (6). The higher enzyme activity in the seeds than in the pods and higher concentrations of ureides in pods than seeds suggests that ureides accumulated in pods may be partly assimilated and partly translocated to the seeds where they are rapidly assimilated. In maturing seeds highest specific allantoinase activities were measured in the embryo axis. A similar distribution of allantoinase activity within a legume seed was reported for germinating seeds of *Lathyrus sativus* (14).

The concentration of allantoin in developing leaves was generally less than 1 μmol/g fresh weight or approximately 1 mM. Allantoinase extracted from soybean seeds has been reported to have a K_m for allantoin of 6.7 (4) and 14 mM (23). The partially purified enzyme from leaf extracts has a K_m of the same order (Thomas and Schrader, unpublished). From these findings it seems that the enzyme may not be operating at maximum efficiency in the leaf or there may be compartmentation of allantoin and allantoinase within some organelle.

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