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^{15}N and ^{13}C NMR Determination of Allantoin Metabolism in Developing Soybean Cotyledons[1]

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ABSTRACT

The metabolism of allantoin by immature cotyledons of soybean (*Glycine max* L. cv Elf) grown in culture was investigated using solid state ^{13}C and ^{15}N nuclear magnetic resonance. All of the nitrogens of allantoin were incorporated into protein in a manner similar to that of each other and to the amide nitrogen of glutamine. The C-2 of allantoin was not incorporated into cellular material; presumably it was lost as CO_2. About 50% of the C-5 of allantoin was incorporated into cellular material as a methylene carbon; the other 50% was presumably also lost as CO_2. The ^{13}C-^{15}N bonds of [5-^{13}C;1-^{15}N] and [2-^{13}C;1,3-^{15}N]allantoin were broken prior to the incorporation of the nitrogens into protein. These data are consistent with allantoin's degradation to two molecules of urea and one two-carbon fragment. Cotyledons grown on allantoin as a source of nitrogen accumulated 21% of the nitrogen of cotyledons grown on glutamine. Only 50% of the nitrogen of the degraded allantoin was incorporated into the cotyledon as organic nitrogen; the other 50% was recovered as NH_4^+ in the media in which the cotyledons had been grown. The latter results suggests that the lower accumulation of nitrogen by cotyledons grown on allantoin was in part due to failure to assimilate NH_4^+ produced from allantoin. The seed coats had a higher activity of glutamine synthetase and a higher rate of allantoin degradation than cotyledons indicating that seed coats play an important role in the assimilation and degradation of allantoin.

The synthesis of storage protein in a developing embryo is dependent upon the flow of nitrogenous compounds to the immature seed from other parts of the plant and the subsequent transfer of nitrogen from these compounds to amino acids. The ureides, allantoin and allantoic acid, are the major forms of nitrogen transported from the root nodules of N_2-fixing soybeans to the shoot (7). At least 35% of the nitrogen arriving in the pod is in the form of ureides (1, 6). Therefore, the pod or the seed must be equipped to degrade the allantoin and incorporate the resulting nitrogen into amino acids.

Allantoinase, the enzyme that converts allantoin to allantoic acid has been isolated from soybeans (19); however, it is not known how allantoic acid is degraded. Based on work with microorganisms, there are several possible pathways by which allantoic acid may be degraded (18). These include (a) the synthesis of glycine plus urea, NH_4^+, and CO_2; (b) the carbamylation of ornithine plus the production of a two-carbon fragment and urea; or (c) the synthesis of two ureas and a two-carbon fragment.

The metabolism of developing soybean cotyledons has previously been examined by incubating immature cotyledons in a culture medium containing ^{13}C;^{15}N-labeled amino acids (14–16). After allowing the cotyledons to grow for 7 to 14 d, they were removed and analyzed by solid state NMR[3]. Analysis by NMR has several advantages over other methods. The NMR analysis can be performed on intact cotyledons, thereby avoiding the elaborate extraction and derivatization procedures normally required in stable isotope studies. Analysis by NMR has the further advantage in that, if ^{13}C;^{15}N double-labeled compounds are fed to the cotyledons, the degree to which ^{13}C-^{15}N bonds remain intact as the compound is metabolized can be measured (12, 13).

In this paper, we report the use of [^{13}C;^{15}N]allantoin, solid state CPMAS, and DCPMAS NMR to study the metabolism of allantoin in developing cotyledons of soybeans. These experiments enabled us to determine if any of the three pathways outlined above was in use. If glycine were formed directly from the carbon and nitrogen of allantoin, we expected that C-5 of allantoin would remain bonded to either N-1 or N-6 of allantoin (see Fig. 1 for numbering of allantoin). If ornithine were carbamylated to form arginine, then the bond between the C-2 and N-1 or between C-2 and N-3 should remain intact. On the other hand, if allantoin were degraded to a two-carbon fragment and urea, which would be hydrolyzed to NH_4^+ and CO_2, then all the C and N bonds of allantoin would be broken. Using DCPMAS ^{15}N NMR, we were able to show that all C-N bonds are broken when cotyledons metabolized either [5-^{13}C;1-^{15}N] or [2-^{13}C;1,3-^{15}N]allantoin.

MATERIALS AND METHODS

Growth of Plants. *Glycine max* (cv Elf) seeds were sterilized by soaking them in a solution containing 0.1% SDS and 10% commercial bleach for 10 min. After they were rinsed in H_2O, they were soaked in a culture of *Rhizobium japonicum* USDA 3Ilb 110 (R. Griffin, Beltsville, MD). The seeds were then planted in 20-cm pots filled with Perlite. Each pot was irrigated daily with 1L of nitrogen-free nutrient solution which contained (in mg/l): $MgSO_4 \cdot 7H_2O$ (480), KH_2PO_4 (135), $CaCl_2 \cdot 2H_2O$ (80), Sequestrene (4.0), H_3BO_3 (0.250), $ZnSO_4 \cdot 7H_2O$ (0.050), $MnSO_4 \cdot H_2O$ (0.170), $CuSO_4$ (0.013), $Na_2MoO_4 \cdot 2H_2O$ (0.010), and $CoCl_2 \cdot 6H_2O$ (0.048). The pH of the final solution was 6.0. Once a week, 5 g of solid $CaSO_4 \cdot 2H_2O$ and 1.4 g of K_2SO_4 were sprinkled on top of the Perlite in each pot. The plants were maintained in a greenhouse with natural light supplemented with

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[3] Abbreviations: NMR, nuclear magnetic resonance; CPMAS, cross-polarization magic-angle spinning; DCPMAS, double cross-polarization magic-angle spinning; GDH, glutamate dehydrogenase; GS, glutamine synthetase.

artificial lights on a 16-h photoperiod. On weekends, the pots were flushed wth H_2O to prevent the build up of salts. Between 70 and 90 d after planting, pods were removed and the immature seeds were used to initiate organ cultures of cotyledons.

Labeled Compounds. The [amide-^{15}N]glutamine (95 atom% ^{15}N) was purchased from Bio-Rad. The [2-^{13}C;1,3-^{15}N]allantoin (90 atom% ^{13}C; 95 atom% ^{15}N), [5-^{13}C;1-^{15}N]allantoin (90 atom% ^{13}C; 95 atom% ^{15}N) and [^{15}N]$(NH_4)_2SO_4$ (98 atom%) were purchased from Merck, Sharpe, and Dohme (Stable Isotopes Division; Montreal, Canada). The [2,7-^{14}C]allantoin (12.5 mCi/mmol) was purchased from Amersham Co. The [U-^{15}N] allantoin (66.9 atom%) was a gift from P. McClure (Los Alamos, NM).

Growth of Cotyledons in Culture. The procedure for growing cotyledons in culture in media containing specific nitrogen sources was based on the procedures of Thompson *et al.* (20) and Skokut *et al.* (15). The media were prepared as given by Skokut *et al.* (15) except the media were sterilized by passage through a 0.2 μm filter. After 14 d the cotyledons were removed, weighed, and lyophilized. The initial fresh weight of the cotyledons was between 8 and 35 mg.

Nitrogen and Carbon Analysis. After the cotyledons were lyophilized, they were crushed with a glass stirring rod. A 2 to 4 mg portion was analyzed using a microKjeldahl procedure (7) with the following modifications. The sodium thiosulfate was omitted and a selenium catalyst (200 mg/20 ml H_2SO_4) was used instead of the zirconium catalyst. The resulting NH_4^+ was analyzed by the hypochlorite-nitroprusside reaction (2). Total carbon was determined by burning a 1 to 2 mg sample with a Carlo Erba analyzer and determining the CO_2 liberated.

Determination of NH_4^+ in Media. Ammonia in media and in cotyledons was analyzed enzymically with GDH (5). Allantoin did not interfere with the assay of NH_4^+ using the enzymic assay whereas it did interfere with the colorimetric assay.

Determination of ^{15}N of NH_4^+ in Media. After the cotyledons were removed, the NH_4^+ from 10 ml of media was distilled from a sodium borate buffer and trapped in a boric acid buffer (21). The NH_4^+ was converted to N_2 by reaction with hypobromite and the ^{15}N content was measured with a VG micromass 602E mass spectrometer.

NMR. Magic-angle spinning ^{15}N NMR spectra were obtained at 20.3 MHz using matched spin-lock cross-polarization transfers with 1-ms contacts and 35 kHz H_1s. The dried samples were contained in a cylindrical double-bearing rotor spinning at 3.2 kHz. Technical details of the spinning and cross-polarization procedures are reported elsewhere (13, 14). Fast cross-polarization rates for protonated nitrogens, long proton rotating-frame lifetimes, and high concentrations of protons in these biological samples ensure representative relative NMR intensities for all nitrogens with a 1-ms contact. The one exception is nitrogen in the form of ammonia or ammonium ion. Internal molecular motion significantly decreases the cross-polarization transfer rates, resulting in signal intensities which underestimate the ammonium nitrogen present. The amount of NH_4^+ in cotyledons was estimated based on the relative signal intensity for NH_4^+ compared to that for one sample for which the NH_4^+ was measured enzymically. Concentrations of other types of ^{15}N in the intact cotyledons were determined quantitatively by comparisons to ^{15}N NMR spectra of a glutamine standard containing a known amount of ^{15}N. Intensities of analytical and calibration spectra were adjusted for differences in rotating-frame proton relaxation rates by the standard procedure of systematic variation of the contact time (17). Magic-angle ^{13}C NMR spectra were obtained at 15.1 MHz using 2-ms cross-polarization transfers from protons and 50 kHz H_1s, with the dried samples contained in a KEL-F Beams-Andrew 700 μl hollow rotor spinning at 2.0 kHz.

Double cross-polarization ^{15}N NMR spectra were obtained using matched spin-lock transfers first from ^{1}H to ^{15}N and then from ^{15}N to ^{13}C. If the ^{13}C-rf field is on resonance and its amplitude satisfies a carbon-nitrogen Hartmann-Hahn condition, a spin-lock transfer from ^{15}N to ^{13}C drains polarization from ^{15}N. A direct difference experiment between single and double cross-polarization procedures then results in the accumulation of a DCPMAS difference ^{15}N signal arising exclusively from the ^{15}N directly bonded to ^{13}C. The fraction of ^{15}N with ^{13}C neighbors can be determined quantitatively from such difference spectra (14).

Determination of GS and GDH Activities. Cotyledons or seedcoats were weighed and 100 to 700 mg were placed in 15-ml polypropylene centrifuge tubes. The tissue was ground with a glass stirring rod after adding 1:2 (w/v) of a solution containing 25 mM Tris-Cl (pH 7.6), 10 mM DTT, and 1 mM $MgCl_2$. After the tissues were ground, additional buffer was added such that the ratio of tissue to buffer was about 1:5 (w/v). The contents were centrifuged at 39,000*g* for 30 min. The activity of GS was assayed by determining the amount of glutamyl-hydroxamate formed in the presence of $MgCl_2$ (11). To assay for GDH activity, 1 to 3 ml of the centrifuged extract was desalted with a Sephadex G-25 column. Two hundred μl of the extract were added to 300

Table I. *Growth and Accumulation of Nitrogen of Cotyledons Grown on Different Sources of Nitrogen*

Immature cotyledons were removed from 75- to 90-d-old plants and placed in media containing either asparagine, glutamine, allantoin, or in various combinations with each compound at a concentration of 15 mM or in media containing $(NH_4)_2SO_4$ at a concentration of 2.5 mM. After 14 d, the cotyledons were removed, lyophilized, and weighed. Total nitrogen was determined by Kjeldahl analysis. *In situ* refers to seeds on the plant of the same maturity as those used to start organ cultures and harvested at the same time as the cultured cotyledons.

Media	Increase in Dry Wt	Increase in Total Nitrogen
	%	
Glutamine	100[a]	100
Allantoin	55	21
Asparagine	79	58
−Nitrogen	46	6
Allantoin + asparagine	66	53
Allantoin + glutamine	85	83
$(NH_4)_2SO_4$	55	6
In situ	39	35

[a] The cotyledons grown on glutamine increased in dry weight 13.7 times and accumulated 0.69 mg nitrogen per cotyledon.

μl of a solution containing 37.5 mM KH_2PO_4 (pH 7.6), 0.24 mM NADH, 10 mM 2-oxoglutarate, and 5 mM NH_4Cl; the production of NAD^+ was monitored spectrophotometrically.

Determination of Rate of Allantoin Degradation. Leaves and podwalls were cut into pieces about 1 × 4 mm. Cotyledons of seeds that weighed 60 to 100 mg were sliced into two pieces along the long axis. Pieces of leaves, cotyledons, and seedcoats (10–40 mg) and podwalls (50–90 mg) were placed into glass scintillation vials. After the fresh weight of the tissue was determined, 700 μl of a solution containing 1 mM [2,7-^{14}C]allantoin (specific activity, 1.35 × 10^3 dpm/nmol) and 15 μM units of urease (type IX, Sigma) were added to each vial. The tissues were vacuum infiltrated for 5 min. One-half of the duplicate vials were sealed with a screw-cap and the other half were sealed with serum caps through which center well cups (Kontes Glass, Vineland, NJ) were suspended. The vials were incubated in the dark while being gently agitated. At the end of 4 h, the tissues in the vials sealed with a screwcap were removed, rinsed with H_2O, patted dry, frozen with dry ice, and lyophilized. These tissues were used to determine the amount of [^{14}C]allantoin taken up. To each of the other vials, 100 μl of 6 N HCl were added per vial and 250 μl of methylbenzethonium hydroxide (Sigma) were added to each center well cup to trap the $^{14}CO_2$. The vials were gently agitated for 1 h, at the end of which time the center wells were removed and placed into separate scintillation vials. Ten ml of scintillation fluid, 3A7 (RPI, Mount Prospect, IL) were added and the $^{14}CO_2$ was determined by liquid scintillation spectrometry.

Although [^{14}C]allantoin as purchased from Amersham did not contain [^{14}C]urea, storage at −20°C resulted in the appearance of [^{14}C]urea within 3 months. Because relatively low levels of [^{14}C]urea (0.2%) interfere with the assay for allantoin degradation, all the assay vials had urease added to them. The amount of contaminating [^{14}C]urea was determined by incubating 700 μl of the 1 mM [^{14}C]allantoin with urease alone.

Determination of Uptake of [^{14}C]Allantoin. The tissues that had been incubated for 4 h in 1 mM [^{14}C]allantoin and lyophilized were extracted at room temperature with $CHCl_3$:CH_3OH:H_2O (5:12:1, v/v/v) (3). The aqueous fraction was dried and redissolved in 0.5 ml H_2O. Aliquots of the aqueous and chloroform extracts were added to a scintillation vial and the radioactivity determined by liquid scintillation spectrometry.

RESULTS

Growth of Cotyledons. Immature soybean cotyledons grew best with 15 mM glutamine as a source of nitrogen (Table I). The growth and accumulation of nitrogen of cotyledons grown with 15 mM allantoin as a source of nitrogen were 55% and 21%, respectively, of that of the cotyledons grown on glutamine. The cotyledons grown on allantoin accumulated about two-thirds the nitrogen of the cotyledons that remained on the plant. The rate of growth and accumulation of nitrogen of cotyledons grown on allantoin or on allantoic acid were not significantly different. The cotyledons grown with asparagine as a sole source of nitrogen accumulated 1.7 times the amount of nitrogen as those cotyledons that remained on the plants. Unexpectedly, cotyledons grown with 5 mM NH_4^+ as source of nitrogen increased in dry weight but did not accumulate nitrogen. (This concentration of NH_4^+ was used because it was the highest concentration of NH_4^+ present in media of cotyledons grown with allantoin as a sole source of nitrogen. See below.) The growth of cotyledons and accumulation of nitrogen were lower when allantoin was included with glutamine or asparagine than when glutamine or asparagine were used as sole sources of nitrogen. Presumed products of allantoin degradation were tested for their effects on the growth of the cotyledons. Glyoxylate (2 mM) had no effect on growth or accumulation of nitrogen when glutamine was used as a source of nitrogen. Cotyledons did not grow when urea (5 mM) was the sole source of nitrogen.

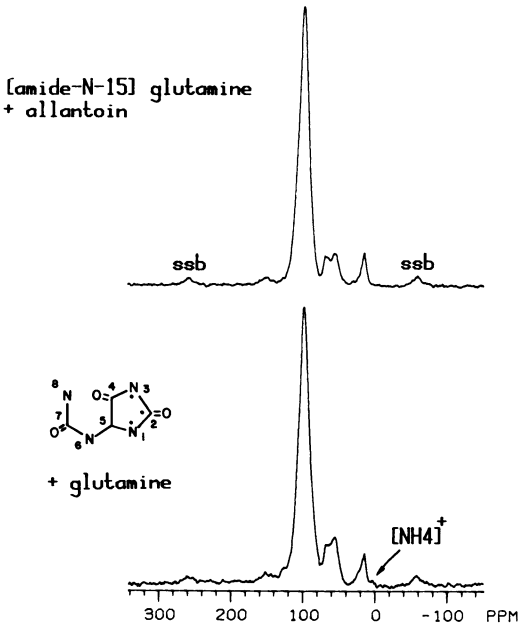

FIG. 1. CPMAS 20.3 MHz ^{15}N NMR spectra of lyophilized cotyledons cultured 14 d on a medium containing either 15 mM [2-^{13}C;1,3-^{15}N]allantoin plus 15 mM glutamine (bottom) or 15 mM [amide-^{15}N] glutamine plus 15 mM allantoin (top). The large central peak (100 ppm) represents ^{15}N present as amide nitrogen (peptide nitrogen of protein plus amide nitrogen of glutamine and asparagine). The small peak at the left of the amide peak (140 ppm) arises from nitrogen of histidine rings. The double peak immediately to the right of the amide peak (60 ppm) represents the guanidino-nitrogens of arginine. The peak to the immediate right of the arginine peak (10 ppm) represents the α-nitrogen of lysine. The shoulder on the lysine peak (20 ppm) arises from the α-amino groups of free amino acids. Ammonium ions, when present, appear at zero ppm, relative to external ammonium sulfate. The remaining two peaks at the high-field and low-field extremes of the spectrum result from the mechanical spinning procedure and are called spinning sidebands (ssb).

^{15}N NMR. Having established that immature cotyledons would grow using the allantoin as a source of nitrogen, we explored how the nitrogen of allantoin was used by immature cotyledons grown in media containing labeled glutamine or allantoin. After 14 d, the cotyledons were lyophilized and analyzed by solid state NMR. The ^{15}N NMR spectra of cotyledons grown on 15 mM [2-^{13}C;1,3-^{15}N]allantoin plus 15 mM glutamine and that of cotyledons grown on 15 mM [amide-^{15}N]glutamine plus 15 mM allantoin were essentially identical (Fig. 1). In addition, there were no major differences in the incorporation of the various nitrogen of allantoin into organic nitrogen from [5-^{13}C;1-^{15}N] and [U-^{15}N]allantoin (Table II). These results demonstrate that all of the nitrogens of allantoin were equivalent and that they were incorporated into protein in a manner similar to that of the amide nitrogen of glutamine. The presence of NH_4^+ or asparagine did not affect the distribution of the nitrogen of allantoin within the organic nitrogen of the cotyledon (Table II). Low levels of $^{15}NH_4^+$ were sometimes detected in cotyledons incubated with [^{15}N]allantoin.

The cotyledons grown on [5-^{13}C;1-^{15}N]allantoin were analyzed by DCPMAS ^{15}N NMR to determine if the ^{15}N-^{13}C bond present in the original allantoin was broken. Several spectra are obtained from this experiment. The first (Fig. 2, bottom) arises from all the ^{15}N present in the sample and is produced by the usual CPMAS process. This spectrum is similar to the ^{15}N NMR

Table II. *Incorporation of ^{15}N into Organic Nitrogen*

Immature cotyledons were grown in media containing either asparagine, glutamine, allantoin, or in combination with each at concentrations of 15 mM or $(NH_4)_2SO_4$ at a concentration of 2.5 mM. After 14 d, the cotyledons were removed, lyophilized, and then analyzed by ^{15}N NMR. The areas of the peaks were determined by digital integration. See Figure 1 for the chemical shifts of the peaks.

Media	Distribution of Incorporated ^{15}N			
	Histidine	Amide	Guanidino	Amino
	% of organic ^{15}N			
[2-^{13}C;1,3-^{15}N] Allantoin	1	81	11	7
[2-^{13}C;1,3-^{15}N] + Allantoin glutamine	1	79	13	7
[amide-^{15}N] Glutamine + allantoin	1	81	13	6
[2-^{13}C;1,3-^{15}N] Allantoin + asparagine	1	78	15	7
[2-^{13}C;1,3-^{15}N] Allantoin + $(NH_4)_2SO_4$	3	80	13	4
$(^{15}NH_4)_2SO_4$ + allantoin	2	85	9	4
[5-^{13}C;1-^{15}N] Allantoin	0	75	16	9
[U-^{15}N] Allantoin	2	84	9	5

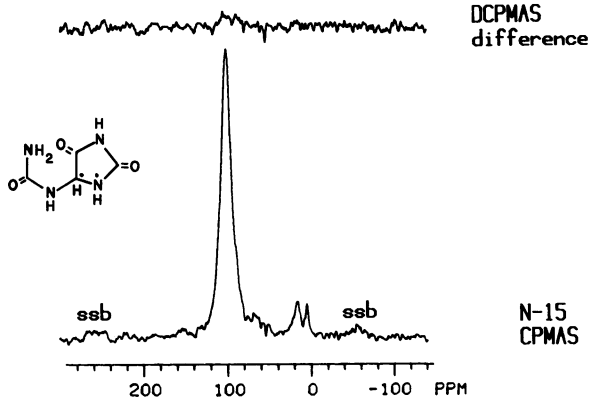

FIG. 2. DCPMAS 20.3 MHz spectrum (top) of intact lyophilized cotyledons cultured for 14 d on a medium containing 15 mM [5-^{13}C;1-^{15}N]allantoin. The top spectrum represents only ^{15}N that is bonded to ^{13}C, and is generated by a double-cross polarization difference technique. The bottom spectrum is similar to those presented in Figure 1.

spectrum obtained from cotyledons grown with [2-^{13}C;1,3-^{15}N] allantoin, showing no preferential routing of nitrogen, as discussed above. (The NH_4^+ peak present in Figure 2 (0 ppm) was usually present in spectra from cotyledons incubated with [2-^{13}C;1,3-^{15}N]allantoin as well, although the level in Figure 1 is atypically low.) The DCPMAS difference spectrum (Fig. 2, top) arises only from those ^{15}N directly bonded to ^{13}C. There are no sizeable peaks in this difference spectrum. This demonstrates the absence of ^{15}N-^{13}C bonds, and the low concentration of free allantoin in the cotyledons (determined independently as 10 nmol/mg dry weight). Similar results were obtained when cotyledons grown on [2-^{13}C;1,3-^{15}N]allantoin were analyzed by DCPMAS NMR.

A nitrogen balance sheet was constructed for cotyledons based on quantitation of ^{15}N as analyzed by NMR and of total nitrogen by Kjeldahl analysis (Table III). The presence of glutamine or asparagine did not affect the incorporation of nitrogen from allantoin when expressed as nitrogen incorporated per mg dry weight. About one-half of the nitrogen of allantoin that was degraded was recovered as NH_4^+ in the medium. The ^{15}N content of the NH_4^+ in the media was on average 96% of the ^{15}N content of the [^{15}N]allantoin used as a source of nitrogen regardless of whether the ^{15}N was in position 1 and 3, 3 alone, or 1, 3, 6, and 8. The presence of glutamine did not affect the percentage of allantoin nitrogen that was recovered as NH_4^+. Upon the addition of NH_4^+, the incorporation of allantoin nitrogen decreased to 70% of those grown on allantoin alone, but the largest percentage of organic nitrogen was still derived from allantoin.

Enzyme Activities. In view of the high percentage of allantoin nitrogen ending up in the media as NH_4^+, we measured the activities of several enzymes to determine if the cotyledons were limited in their ability to assimilate NH_4^+. The activity of GDH of cotyledons in the presence of 5 mM NH_4^+ was 1.5 to 4.8 nmol glutamate synthesized/mg fresh weight·h (Table IV). The level of GDH in cotyledons grown on allantoin was 27% higher than cotyledons grown on glutamine. GDH was about equally distributed between seed coats and cotyledons. The levels of GDH in cotyledons from the plant increased as seeds aged. The K_m for NH_4^+ of GDH under the conditions listed in "Materials and Methods" was 40 mM. The enzyme was assayed in the presence of 5 mm NH_4^+ as this was the highest NH_4^+ concentration present in the media of cotyledons grown on allantoin. GS activity of crude extracts of cotyledons varied between 0.55 and 0.95 nmol glutamine synthesized/mg fresh weight·h. The activity of GS in the seed coats was 7 to 12 times higher than the activity in cotyledon extracts when expressed on a mg fresh weight basis and 2 to 7 times higher when expressed on per seed basis.

The possibility that the seed coat plays a role in the degradation of allantoin was investigated by determining the relative rates of uptake of [2,7-^{14}C]allantoin and the release of $^{14}CO_2$ from various tissues (Table V). The seed coats degraded allantoin to CO_2 about 4 times faster than the cotyledons and 1.3 times faster than leaves. The podwalls did not degrade allantoin to CO_2.

^{13}C NMR. The cotyledons were also analyzed by ^{13}C NMR to determine the fate of the carbons of allantoin. The ^{13}C NMR spectrum of cotyledons grown with ^{13}C natural abundance allantoin and that of cotyledons grown with [2-^{13}C]allantoin were identical, demonstrating that the C-2 of allantoin was completely lost (Fig. 3, top row). In contrast, the spectrum obtained from cotyledons grown with [5-^{13}C]allantoin differs from that obtained from cotyledons grown with ^{13}C natural abundance allantoin. About 0.26 μmol/mg dry weight, of the carbon from C-5, was retained within the cotyledons. This represents 46% of the allantoin that was metabolized by these cotyledons based on the incorporation of allantoin nitrogen. From the chemical shifts, the C-5 of allantoin was retained as a methylene carbon in protein (Fig. 3, bottom left, 60 ppm region).

Table III. *Distribution of Allantoin Nitrogen*

Immature cotyledons were cultured for 14 d on media containing 15 mM [2-^{13}C;1,3-^{15}N]allantoin and where indicated 15 mM glutamine or asparagine or 2.5 mM $(NH_4)_2SO_4$. The organic nitrogen accumulated was determined by subtracting the nitrogen initially present in duplicate seeds from the nitrogen in cotyledons grown in culture as measured by Kjeldahl analysis. The incorporation of allantoin nitrogen into organic nitrogen was determined by ^{15}N NMR analysis of the intact lyophilized cotyledons. The allantoin nitrogen recovered in the media as NH_4^+ was quantified by enzymatic analysis.

	Allantoin N Incorporated into Organic N/mg Dry Wt	Organic N Derived from Allantoin N	Allantoin N Recovered as NH_4^+
	% of allantoin-grown cotyledons	*% of organic N*	*% of N formed from allantoin*
Allantoin	100[a]	106	48
Allantoin + glutamine	103	55	41
Allantoin + asparagine	98	35	
Allantoin + $(NH_4)_2SO_4$	70	82	

[a] The average value for cotyledons grown on allantoin as a sole source of nitrogen was 22 μg N/mg dry weight.

Table IV. *Levels of GDH and GS in Cotyledons and Seed Coats*

Immature seeds were harvested and assayed immediately (A), or were cultured on media containing 15 mM glutamine or 15 mM allantoin and assayed after 7 d in culture (B). GS was assayed by determining glutamylhydroxamate. GDH was assayed by the production of NAD^+.

Tissue	GDH	GS	GDH	GS
	nmol/mg fresh wt·h		*nmol/seed·h*	
A. Cotyledon (seed wt, 20–60 mg)	1.5	0.55	52	16
Seed coat	2.8	6.69	51	110
Cotyledon (seed wt, 100–200 mg)	4.8	0.85	325	94
Seed coat	2.6	5.86	82	199
B. Cultured cotyledons				
0 d		0.95		
7 d				
Glutamine media	1.5	0.95		
Allantoin media	1.9	1.10		

Table V. *Uptake and Degradation of Allantoin by Different Tissues*

Pieces of the indicated tissues were incubated in the dark 4 h in 1 mM [2,7-^{14}C]allantoin (specific activity, 1.23×10^3 dpm/nmol). Degradation was determined by trapping the $^{14}CO_2$ that was released with methylbenzethonium hydroxide. The $^{14}CO_2$ thus trapped was determined by liquid scintillation spectrometry. Duplicate samples to those used for determining rate of degradation were removed from the vials after the 4-h incubation and lyophilized. The lyophilized samples were extracted and the ^{14}C was determined by liquid scintillation spectrometry. Only background levels of ^{14}C were present in the chloroform extract. Uptake was determined by adding the dpm released as CO_2 to those dpm still remaining in the tissue.

Tissue	Allantoin Uptake	Allantoin Degradation
	dpm ^{14}C/mg fresh wt·4 h	
Cotyledon	926	296
Seed coat	2628	1200
Leaf	4280	906
Podwall	1850	≤10

Cotyledons grown without a source of nitrogen accumulated 46% of the dry weight of cotyledons grown on glutamine, but only 6% of the nitrogen. The peaks arising from carboxyl (180 ppm) and methylene (30 ppm) carbons of protein are absent from the ^{13}C spectrum of these cotyledons (Fig. 3, bottom right), whereas the peaks due to carbohydrate carbons (75–105 ppm) are present. This confirms that protein was not accumulated and the increase in dry weight was the result of an increase in carbohydrates.

DISCUSSION

Allantoin Degradation. We have demonstrated that cotyledons degrade allantoin and utilize the resulting nitrogen for protein synthesis. The evidence presented in Table II and Figures 2 and 3 is consistent with the degradation of allantoin into two ureas and one two-carbon fragment (1, 14, 18). The loss of ^{13}C from cotyledons incubated with [2-^{13}C]allantoin and the production of $^{14}CO_2$ from cotyledons incubated with [2,7-^{14}C]allantoin is consistent with the loss of carbon as CO_2 during the hydrolysis of urea. The appearance of $^{15}NH_4^+$ (Fig. 2) in cotyledons grown with [^{15}N]allantoin and the similar ^{15}N NMR spectra obtained from cotyledons grown with [^{15}N]allantoin or [^{15}N]glutamine demonstrates that allantoin was converted to NH_4^+ and further suggests that this NH_4^+ was assimilated by GS and glutamate synthase.

The retention of ^{13}C in cotyledons incubated with [5-^{13}C] allantoin also indicates that a two-carbon fragment, such as glyoxylate, was produced from the C-4 and C-5 of allantoin. This two-carbon fragment was then used for the synthesis of other cellular constituents (Fig. 3, bottom left). We failed, however, to detect tissue-dependent production of labeled urea or ureidoglyoxylate from [2,7-^{14}C]allantoin (unpublished observations). Although we did not establish the exact pathway by which allantoin was degraded, certain pathways mentioned in the introduction, such as the carbamylation of ornithine and the specific synthesis of glycine from the carbon skeleton and one of the nitrogens of allantoin, can be eliminated.

Nitrogen Utilization. Cotyledons grown on allantoin did not accumulate as much nitrogen as those cotyledons grown on

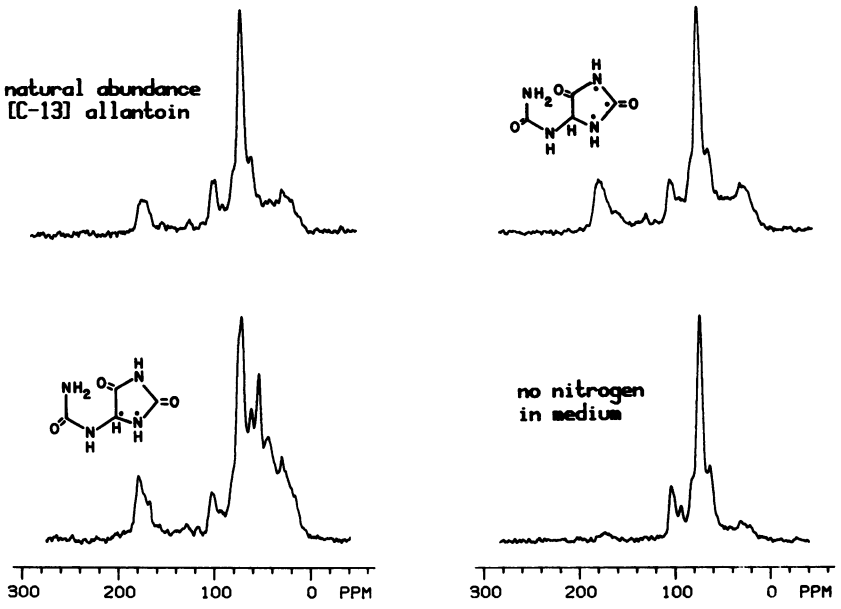

FIG. 3. CPMAS 15.1 MHz ^{13}C NMR spectra of intact lyophilized cotyledons cultured 14 d on media containing 15 mM ^{13}C natural abundance allantoin (top, left), [2-^{13}C;1,3-^{15}N]allantoin (top, right), [5-^{13}C;1-^{15}N]allantoin (bottom, left), or media without a source of nitrogen (bottom, right). The major peaks represent carbonyl and carboxyl carbons in protein (180 ppm), hydroxylated carbons of polysaccharides (80 and 105 ppm), and methyl and methylene carbons in protein and structural lipid (20–60 ppm).

glutamine or those left on the plant (Table I). The cotyledons did not incorporate all of the NH_4^+ that was produced from the allantoin. About 50% of the NH_4^+ produced from allantoin was recovered as NH_4^+ in the media. The finding that the ^{15}N content of the NH_4^+ in the media was essentially the same as that of the allantoin provided demonstrates that the NH_4^+ was derived from allantoin. It also further demonstrates that all of the nitrogens of allantoin were equivalent in that each had an equivalent chance to end up as NH_4^+ in the media.

Cotyledons did not grow as well when both allantoin and glutamine were present as when glutamine alone was present. This is probably due to increased NH_4^+ concentrations when allantoin was included. The other presumed product of allantoin degradation, glyoxylate, had no effect on growth. The failure of the cotyledons to accumulate nitrogen when 5 mM NH_4^+ was the sole source of nitrogen suggests that NH_4^+, once exported, becomes unavailable to the cotyledon. The failure of the cotyledons to incorporate the NH_4^+ from allantoin before it leaks out into the media can occur because either the levels of GS are insufficient to assimilate the nitrogen, or the rate at which glutamate is produced is insufficient to provide substrate for the assimilation of the NH_4^+ by GS. The cotyledons accumulated 16 to 33 μg nitrogen/mg dry weight·14 d or roughly 0.7 to 1.4 μmol nitrogen/mg fresh weight·h. This rate is of the order of the GS levels in the cotyledons (Table IV) suggesting that the levels of GS are indeed limiting the assimilation of NH_4^+.

There is no failure to assimilate NH_4^+ *in vivo*. Ureides appear to be degraded within the seed coat and the resulting nitrogen used for the synthesis of glutamine. Nitrogen leaving the seed coat exits primarily as glutamine, asparagine, and NH_4^+ (10); ureides do not exit from the seed coat. In peas, the seed coat may play an active role in the nutrition of the cotyledons (8, 9). For example, high levels of asparaginase are present within the seed coat suggesting that asparagine is degraded within the seed coat (9). For soybeans, the presence of higher levels of GS and higher rates of allantoin degradation in the seed coat than in the cotyledons (Table V), supports the view that *in situ* the ureides are degraded and the resulting nitrogen converted into glutamine within the seed coat.

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