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Ureide Catabolism of Soybeans[1]

II. PATHWAY OF CATABOLISM IN INTACT LEAF TISSUE

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ABSTRACT

Allantoin catabolism studies have been extended to intact leaf tissue of soybean (*Glycine max* L. Merr.). Phenyl phosphordiamidate, one of the most potent urease inhibitors known, does not inhibit $^{14}CO_2$ release from [2,7-^{14}C]allantoin (urea labeled), but inhibits urea dependent CO_2 release ≥99.9% under similar conditions. Furthermore, $^{14}CO_2$ and [^{14}C] allantoate are the only detectable products of [2,7-^{14}C]allantoin catabolism. Neither urea nor any other product were detected by analysis on HPLC organic acid or organic base columns although urea and all commercially available metabolites that have been implicated in allantoin and glyoxylate metabolism can be resolved by a combination of these two columns. In contrast, when allantoin was labeled in the two central, nonureido carbons ([4,5-^{14}C]allantoin), its catabolism to [^{14}C]allantoate, $^{14}CO_2$, [^{14}C]glyoxylate, [^{14}C]glycine, and [^{14}C]serine in leaf discs could be detected. These data are fully consistent with the metabolism of allantoate by two amidohydrolase reactions (neither of which is urease) that occur at similar rates to release glyoxylate, which in turn is metabolized via the photorespiratory pathway. This is the first evidence that allantoate is metabolized without urease action to NH_4^+ and CO_2 and that carbons 4 and 5 enter the photorespiratory pathway.

The ureides[2] allantoin and allantoate are the major N compounds transported in the xylem of N_2-fixing soybean plants and several other tropical legumes (1, 8, 19). Despite the importance of these compounds as N metabolites, little is known about ureide catabolism. Allantoinase, the enzyme that catalyzes the hydrolysis of allantoin to allantoate, is abundant in stems, leaves, nodules, and the fruits of soybeans (7, 18, 20, 23). Lee and Roush (7) have reported that the soybean allantoinase is specific for D-Allantoin, while others have reported that L-allantoin is catabolized, but at only 10% the rate of D-allantoin (23). N has been generally assumed to be released from allantoate through urea via urease action (9) but there is insufficient published evidence to support this hypothesis.

Two allantoate catabolizing enzymes are found in microbes (22, 25), allantoate amidohydrolase (ALAH)[3] [EC 3.5.3.9] and allantoate amidinohydrolase [EC 3.5.3.4] (Fig. 1). The amidinohydrolase catalyzes the hydrolysis of allantoate to urea and ureidoglycolate, whereas ALAH, the amidohydrolase, catalyzes the hydrolysis of allantoate to $2NH_4^+$, CO_2 and ureidoglycolate with a proposed ureidoglycine intermediate (22). The subsequent metabolism of ureidoglycine and ureidoglycolate in microorganisms is outlined in Figure 1. The metabolism of glyoxylate, a product of ureidoglycolate, is outlined in Figure 2. The amidinohydrolase is generally termed allantoicase and has been proposed to be present in soybeans (14). On the other hand we have previously demonstrated evidence for ALAH in extracts of developing soybean (27).

Several different reports have focused on plant ALN metabolism, but no report has conclusively elucidated the entire pathway of catabolism. Coker *et al.* (2) found that cultured soybean cotyledons metabolized [^{13}C-^{15}N]ALN so that all C—N bonds were broken before reincorporation into protein. Further, they found that 50% of the No. 5 carbon of allantoin was retained in the tissue as a methylene carbon.

Atkins *et al.* (1) demonstrated the release of $^{14}CO_2$ from cowpea tissue fed [2-^{14}C]ALN, indicating that the ureido-carbonyl bond(s) of allantoin were hydrolyzed to release CO_2. Moreover, they recovered low levels of [^{14}C]urea from aphids feeding on the leaflets and fruits of cowpea that were fed [2-^{14}C]ALN through the transpiration stream. These results are consistent with the action of either allantoicase or allantoate amidohydrolase followed by the subsequent release of urea from ureidoglycolate.

Shelp and Ireland (14) observed that the urease inhibitor and metal chelator (26), AHA, inhibited the release of $^{14}CO_2$ from [2-^{14}C]ALN in soybean leaf tissue. Additionally, they found an accumulation of unlabeled urea in AHA-treated leaf discs fed ALN as compared to discs fed ALN in the absence of AHA. They concluded that ALN metabolism releases 2 urea/ALN via allantoate amidinohydrolase and ureidoglycolase (ureidoglycolate urea-lyase [EC 4.3.2.3]).

Results from our laboratory, however, favor the action of allantoate amidohydrolase, *i.e.* the degradation of allantoate directly to NH_4^+ and CO_2 without the production of a urea intermediate. Polacco *et al.* (12) found that nickel-starved soybean suspension culture cells (therefore lacking an active urease) could grow on ALN, but not on urea. This result strongly suggested that the nitrogen of ALN was at least partially catabolized without urease action. Winkler *et al.* (27) provided evidence for an Mn^{2+}-dependent allantoate amidohydrolase in developing soybeans and seed coats. In experiments where urease was completely inhibited by PPD, (one of the most potent urease inhibitors known [17]) the release of NH_4^+ and CO_2 from allantoate was unaffected. Additionally, urea levels were found to be less than 8% of the CO_2 produced in the presence of PPD, eliminating allantoate amidinohydrolase as the primary catabolic route. All urea production could be explained by

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[2] Ureides here refer only to allantoin and allantoate.

[3] Abbreviations: ALAH, allantoate amidohydrolase; ALN, allantoin; AHA, acetohydroxamic acid; PPD, phenyl phosphordiamidate; Mops, 4-(*N*-morpholine)propanesulfonic acid; OB10, organic base fraction 10.

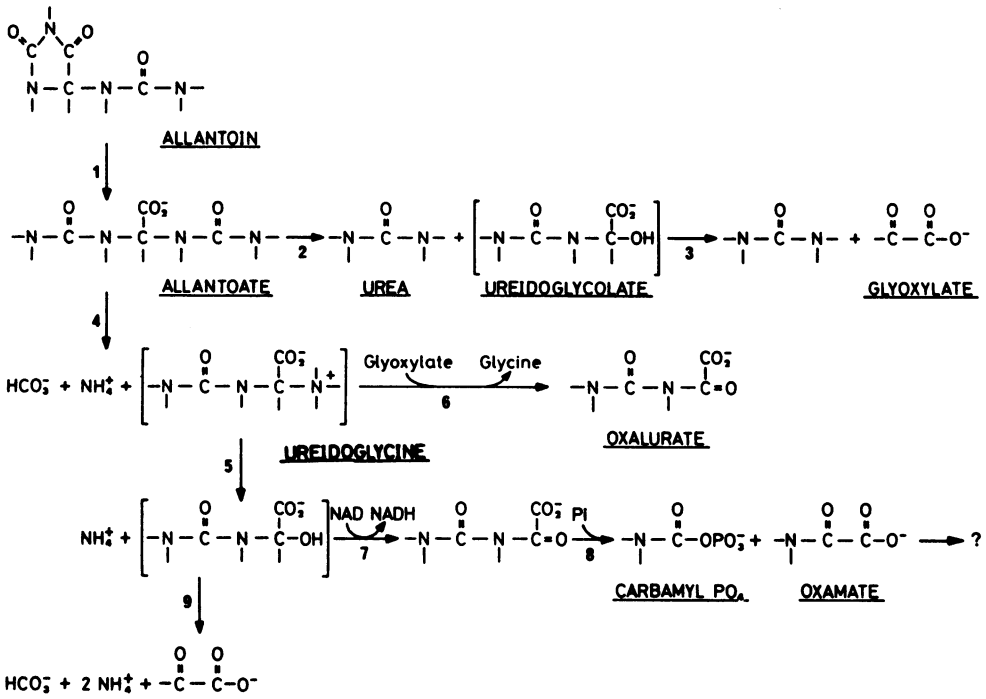

FIG. 1. Catabolic pathways of allantoin assimilation. (1), Allantoinase; (2), allantoate amidinohydrolase; (3), ureidoglycolate urea-lyase; (4), allantoate amidohydrolase; (5), ureidoglycine aminohydrolase; (6), transamination (nonenzymic); (7), ureidoglycolate dehydrogenase; (8), oxamate transcarbamylase; (9), ureidoglycolate amidohydrolase. Step (6), transamination, has been demonstrated *in vitro* but never *in vivo*. There are no reports suggesting ureidoglycolate amidohydrolase other than present data. Taken from Refs. 19, 21, and 25.

nonenzymic degradation of ureidoglycolate or ureidoglycine. Further, we found 2 ethanolthio, 2'ureido, acetic acid [$NH_2CONHCHCO_2HSCH_2CH_2OH$] in partially purified extracts incubated with 2-mercaptoethanol and allantoate, suggesting that a reactive ureido intermediate is produced that reacts with thiols *in vitro*.

This report characterizes the *in vivo* metabolism of allantoin in soybean leaves, and provides evidence for the involvement of two amidohydrolase reactions being responsible for allantoate degradation to NH_4^+, CO_2, and glyoxylate. Evidence concerning the subsequent metabolism of glyoxylate is fully consistent with its metabolism through the photorespiratory cycle.

MATERIALS AND METHODS

Plant Material. Nodulated plants of soybean (*Glycine max* L. Merr. cv Williams 82) inoculated with *Rhizobium japonicum* 3I1B143 were growth chamber grown under a 16:8 h, 34°C:28°C, light:dark regime with light intensity of 400 $\mu E \cdot m^{-2} \cdot s^{-1}$. Seedlings were grown in Perlite in modified Leonard jars containing a nitrogen-free nutrient solution as described previously (20), except that K_2HPO_4 was increased to 0.5 mM. Plants were harvested after 2 to 3 h of light at 4 to 6 weeks of age. A cork borer was used to prepare 63 mm^2 leaf discs from fully expanded trifoliate leaves.

Chemicals and Radiolabel. [2,7-^{14}C]ALN (0.1 mCi/mmol) was synthesized and recrystallized by the procedure of Zellner and Stevens (28). [4,5-^{14}C]ALN (6 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). Purity of [2,7-^{14}C]ALN was ascertained by a combination of urease-dependent urea analysis, HPLC, and TLC. Ten μmol of [2,7-^{14}C]ALN were incubated with 10 units of Jack Bean urease Type VII (Sigma Chemical) for 1 h and $^{14}CO_2$ release was quantitated. Only 0.06% of the [^{14}C] was released as $^{14}CO_2$ by urease. TLC (22) cellulose plates (Sigma) were developed with *n*-butanol/acetic acid/H_2O) (12:3:5). R_F for ALN and urea were 0.36 and 0.56, respectively. The ALN was ≥99.0% pure by autoradiography after a 10 d exposure (20,000 cpm loaded).

[2,7-^{14}C]- and [4,5-^{14}C]ALN were judged ≥99.8% pure by HPLC analysis on an ion exclusion column (BioRad Aminex HPX-87H, 300 × 7.8 mm) with 0.014 N H_2SO_4 as a mobile phase (13, 27). Fractions were collected and quantitated by liquid scintillation spectrometry. Purity ≥99.8% was likewise observed on an organic base column (Bio-Rad Aminex HPX-725 300 × 7.8 mm) with 0.065 N $(NH_4)_2SO_4$ as the mobile phase.

PPD (ICN, Plainview, NY) and ALN (Sigma) were recrystallized from CH_3CN and H_2O, respectively. AHA was purchased from Aldrich Chemicals, (Milwaukee, WI). Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Company.

$^{14}CO_2$Analysis. $^{14}CO_2$ was quantitated by a modification of the methods of Kerr *et al.* (5). Freshly harvested leaf discs (0.1 g) were added to a 2.0 ml solution of 20 to 50 mM Mops (pH 6.8) containing 0.2% (v/v) Tween-20. In assay vials containing PPD, leaf discs were preincubated with PPD in the absence of substrate for 15 to 25 min. The incubation was initiated with 0.14 to 2.0 ml of 20 mM [2,7-^{14}C]ALN. The reaction was stopped by adding 0.5 ml of 5 N H_2SO_4. The $^{14}CO_2$ was trapped on glass fiber filter discs impregnated with 150 μl 20% saturated NaOH (prepared fresh for each assay from a saturated stock). $^{14}CO_2$ was corrected for counting efficiency and trapping efficiency.

Analysis of Intermediates. Leaf discs (50 or 100 mg, 20 mm^2) were incubated with [4,5-^{14}C]ALN or [2,7-^{14}C]ALN in the same buffer as above except that the reaction volume was reduced to 300 or 600 μl, respectively. $^{14}CO_2$ was quantitated as above in separate vials. Controls were samples treated similarly except without leaf tissue. Preliminary experiments established that [^{14}C]glyoxylate could not be recovered from leaf tissue unless unlabeled glyoxylate was included in the extraction buffer. Samples for HPLC analysis were homogenized in a glass homogenizer with 200 μl $CHCl_3$ and 100 μl of 100 mM Mops (pH 7.0), containing 10 mM concentrations of the following: ALN, potassium allantoate, ammonium ureidoglycolate, glyoxylate, oxamate, oxalate, glycine, and serine. Samples were stored frozen and prior to HPLC analysis were centrifuged at 10,000*g* for 5 min and the supernatant (aqueous phase) was injected directly onto a Bio-Rad organic acid (0.7 ml/min with 0.014 N H_2SO_4 as mobile phase) or organic base column (1.0 ml/min with 0.065 M $[NH_4]_2SO_4$ as mobile phase). Fractions were collected and analyzed by liquid scintillation spectrometry. All samples were corrected for counting efficiency.

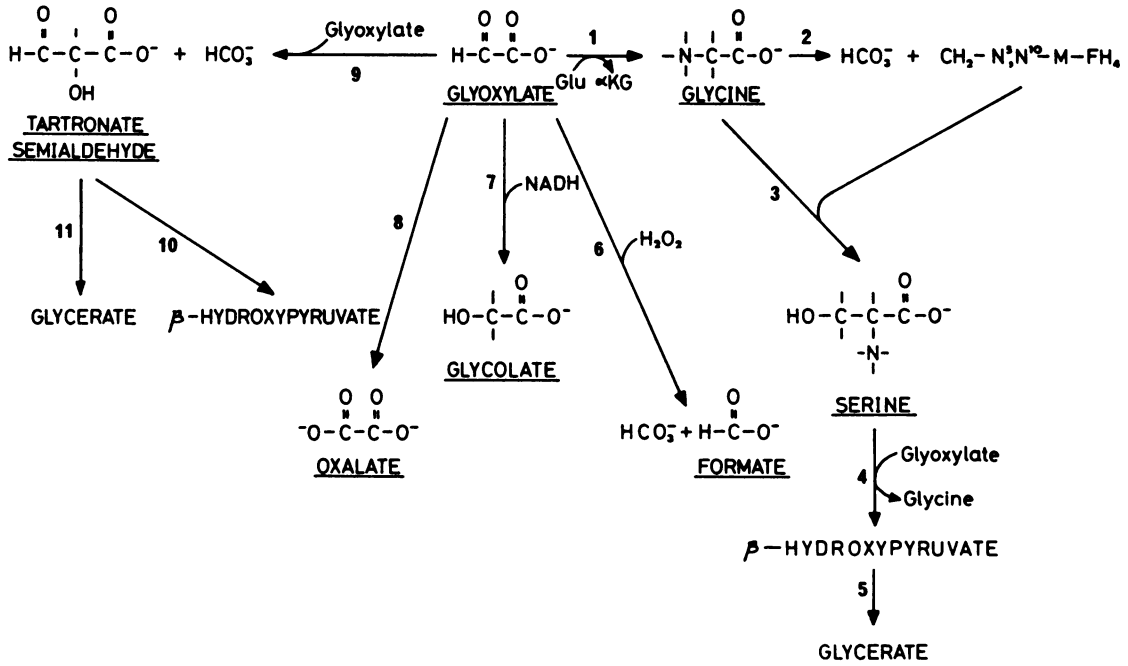

FIG. 2. Potential pathways of catabolism of glyoxylate in both plants and microorganisms. (1), Glutamate-glyoxylate aminotransferase; (2), glycine decarboxylase; (3), serine hydroxymethyltransferase; (4), serine-glyoxylate aminotransferase; (5), D-glycerate dehydrogenase; (6), nonenzymic oxidation of glyoxylate; (7), NADPH-glyoxylate reductase; (8), glyoxylate oxidase; (9), tartronate semialdehyde synthase; (10), isomerase; (11), tartronate semialdehyde reductase. Reactions (1), (2), (3), and (4) are well documented for plants (4). Although reactions (6), (7), and (8) have been proposed to occur in plants they are not known to be primary catabolic pathways. Taken from Refs. 3, 4, 7, 9, 15, and 25.

Carbamyl amino acids were prepared by the method of Stark (16).

TLC of Isolated Labeled Compounds. Fractions (1–2 μl) of the HPLC purified compounds (fraction OB10 was dried under vacuum and redissolved in 10 μl H_2O) were spotted on silica (HP thin layer plates, Pierce, Rockford, IL) or cellulose plates (Sigma) in 0.5 μl aliquots. Silica plates were developed in 95% ethanol/34% NH_4OH 7:3 v/v (10). Cellulose plates were developed in methanol/pyridine/H_2O 20:5:1 v/v (10). Serine, glycine, glutamine, and alanine (1 μl of 1% solution) were spotted as standards and detected with ninhydrin after autoradiography.

RESULTS

CO_2 Production from [2,7-^{14}C]allantoin. Leaf discs incubated with [2,7-^{14}C]ALN generated $^{14}CO_2$ at a linear rate for 1.5 h (Fig. 3) indicating that the ureido-carbonyl bonds of allantoin were being hydrolyzed. These results confirm those reported by others (1, 14).

Inhibition by AHA. AHA at 50 and 100 mM inhibited release of $^{14}CO_2$ from both [^{14}C]urea and [2,7-^{14}C]ALN by >93% (Table I). These results confirm the results reported by Shelp and Ireland (14). AHA, a urease inhibitor and metal chelator (26), also inhibits allantoate-dependent glyoxylate production in partially purified extracts of soybean seed coats (27). Data from our laboratory have established that AHA also inhibits ALAH in extracts of soybean leaves (6). Thus, AHA inhibition of ALN dependent $^{14}CO_2$ release is not an adequate basis for concluding that urea is an intermediate in allantoin catabolism. Indeed, AHA also inhibits NADPH-glyoxylate reductase (6) so that it is obviously not a specific inhibitor of urease.

Inhibition by PPD. PPD is one of the most potent urease inhibitors known (17) with a $K_i < 20$ μM for urease from soybean leaves (5). Preliminary experiments indicated that PPD did not block the uptake of [^{14}C]urea by intact leaf discs, whereas $^{14}CO_2$ release from 50 mM [^{14}C]urea was inhibited >99.9% by 0.8 mM PPD. In contrast, allantoin-dependent $^{14}CO_2$ release is linear and not inhibited by PPD (Fig. 3). Lack of PPD inhibition of the release of CO_2 from ALN indicates that little or no free urea is released from allantoate under these experimental conditions. Such release would be expected if allantoate amidinohydrolase was the primary allantoate catabolizing enzyme in soybean leaf discs. These results are consistent with a pathway that includes allantoate amidohydrolase and a later step that releases the second ureido CO_2 in a urease-independent manner. The only reported example of this (25) is the degradation of oxalurate to oxamate and carbamyl phosphate by oxamic transcarbamylase in a microbial system (Fig. 1). Alternatively, the involvement of ureidoglycolate amidohydrolase action is possible, although there are no reported examples of this activity.

Urease Activity in the Presence of Allantoin. If urease activity were inhibited by the nonphysiological levels of ALN employed, or by one of its metabolites, then the lack of inhibition by PPD would be less conclusive. Urease is not inhibited by 30 mM ALN (Fig. 4) suggesting that urease is active under conditions where ALN is metabolized in intact leaf discs. Low levels of urea were employed to mimic the situation if urea was an intermediate in the catabolism of allantoin.

[$^{14}CO_2$] Release from [4-5-^{14}C]Allantoin. The rate of $^{14}CO_2$ evolution from [4,5-^{14}C]ALN was compared to that from [2,7-^{14}C]ALN (Fig. 5). [4,5-^{14}C]ALN releases $^{14}CO_2$ at approximately 40% the rate that [2,7-^{14}C]ALN releases $^{14}CO_2$, suggesting that 60% of the label should be retained as stable carbon compounds, *e.g.* glyoxylate, glycolate, oxamate, glycine, serine, oxalate, or formate.

Labeling of Allantoin-Derived Metabolites in Intact Tissue. All of the compounds (commercially available) that are known to be involved in microbial ureide metabolism as well as possible associated pathways in plants are listed in table II with their respective retention times on HPLC organic acid and organic base columns. By using both columns in combination with TLC, chemical analysis, and differentially ^{14}C-labeled ALN, all of the known, commercially available catabolites may be identified and quantitated. Unfortunately, allantoate is unstable under the acidic conditions of the organic acid column, so that [^{14}C] glyoxylate (the hydrolysis product of [4,5-^{14}C]allantoate in acid)

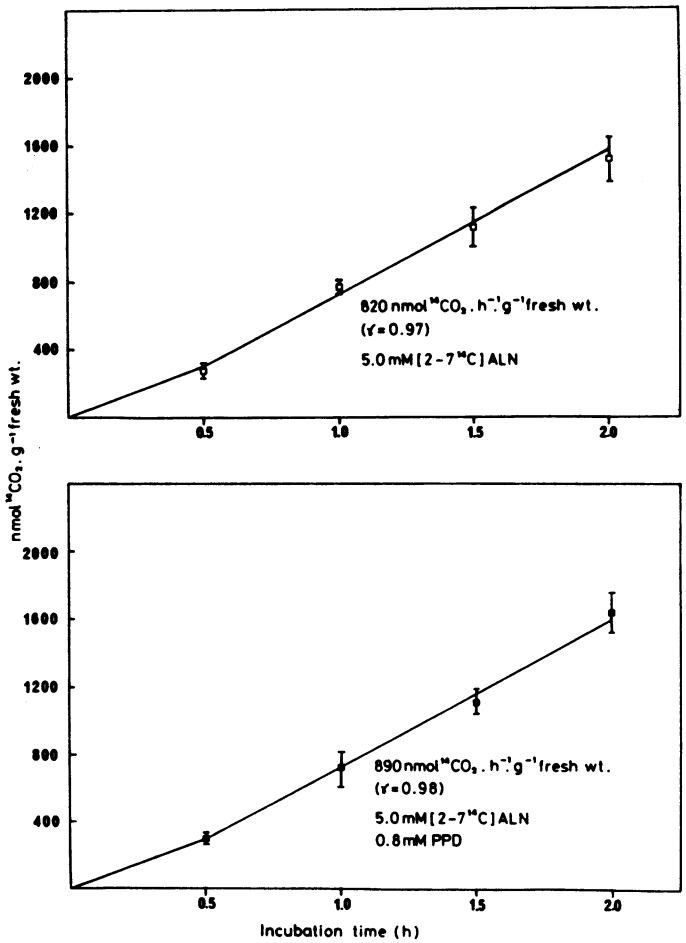

FIG. 3. Effect of PPD on allantoin metabolism in soybean leaf discs. Leaf discs were incubated in buffered (pH 6.8) 5.0 mM [2,7-^{14}C]ALN in the presence (■) or absence (□) of 0.8 mM PPD. $^{14}CO_2$ was trapped on glass fiber filter discs impregnated with 150 μl 25% saturated NaOH and measured by scintillation spectrometry. Error bars represent measurements in quintuplicate.

Table I. *Inhibition of $^{14}CO_2$ release by AHA*

Leaf discs (100 mg) were incubated with 1.3 mM [2,7-^{14}C]allantoin or 33 mM [^{14}C]urea for 2 h. Assays were in triplicate.

	$^{14}CO_2$ Release	
	[^{14}C]Urea	[^{14}C]Allantoin
	μmol·h^{-1}·g^{-1} fresh wt	
No addition	4.4	0.30
50 mM AHA	0.23	0.02
100 mM AHA	0.10	0.00

is observed as a level background from 10.0 min to 20.0 min on chromatograms or in radiolabeled fractions. This is about 20% of the allantoate label and this background increases the detection limit of compounds migrating between glyoxylate and allantoate on the organic acid column. Likewise, [2,7-^{14}C]allantoate was hydrolyzed by the acidic conditions of the organic acid column and gave a level [^{14}C] background. Fractions were collected for 30 min on the organic acid column and for 50 min on the organic base column. Greater than 90 to 95% of the label was recovered in these fractions.

Labeled Products of [2,7-^{14}C]ALN Metabolism. When leaf discs were incubated with [2,7-^{14}C]ALN, the only radiolabeled products detected were $^{14}CO_2$ and [^{14}C]allantoate (Table III). Samples were analyzed for $^{14}CO_2$ and for other products by both

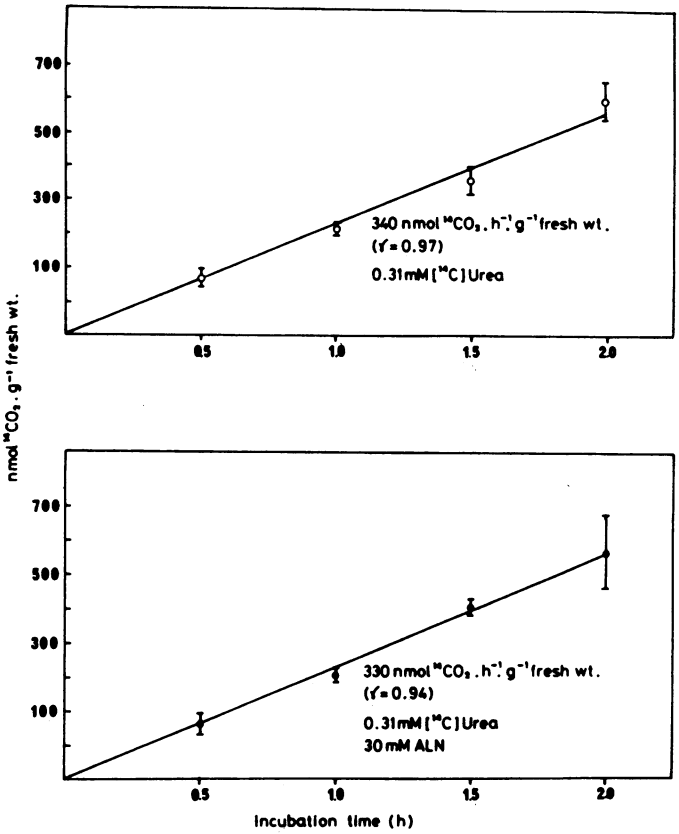

FIG. 4. Effect of allantoin metabolism on urea by soybean leaf discs. Leaf discs were incubated with 0.31 mM [^{14}C]urea in the presence (●) or absence (○) of 30 mM ALN and $^{14}CO_2$ was determined. Error bars represent quadruplicate measurements.

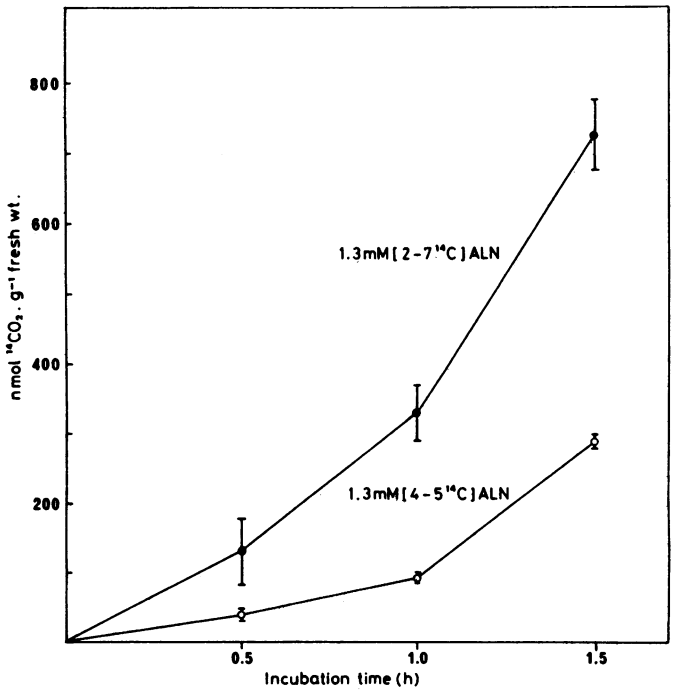

FIG. 5. Comparison of $^{14}CO_2$ release from [4,5-^{14}C]ALN *versus* [2,7-^{14}C]ALN. Leaf discs were incubated with 1.3 mM [2,7-^{14}C]ALN (●) or 1.3 mM [4,5-^{14}C]ALN (○) and $^{14}CO_2$ was determined.

Table II. *HPLC Retention Times of Possible Ureide Metabolites*

Standards were dissolved in H_2O and injected on a Bio-Rad Aminex HPX-87H (300 × 7.8 mm) organic acid column with 0.014 N H_2SO_4 as a mobile phase. Flow rate was 0.7 ml/min. Likewise, standards were injected onto a Bio-Rad Aminex HPX-72S (300 × 7.8 mm) organic base column with 0.065 M $(NH_4)_2SO_4$ as mobile phase and 1 ml/min as the flow rate. Effluent of both columns was monitored at 210 nm with a chart speed of 15 cm/h.

	Organic Acid Column Migration Time	Organic Base Column Migration Time
	min	*min*
ALN	16	21.0
Allantoate	20	36.0
Ureidoglycine	[a]	[a, b]
Ureidoglycolate	13.8	36.0
Oxalurate	7.2	>80.0
Glyoxylate	10.0	36.4[c]
Oxamate	11.3	44.0
Glycolate	12.8	29.0
Oxalate	7.4	>80
Glycine	>80.0	9.0
Serine	>80.0	9.4
Formate	14.25	46.4
Urea	>80.0	12.0
Glycerate	11.2	
Hydroxy-Pyruvate	10.2	
Malate	8.8	
Tartronate semialdehyde	[a]	[a]
Alanine	>80.0	7.8

[a] Not commercially available. [b] A similar compound, albizziin, $NH_2CONHCH_2CHNH_3^+CO_2^-$, is eluted at 9.0 min. [c] Not clearly resolved from allantoate.

Table III. *[^{14}C] Products from [4,5-^{14}C] and [2,7-^{14}C]ALN*

Leaf discs (50 mg) were incubated with 300 μl 1.3 mM [4,5-^{14}C]ALN in pH 6.8 Mops, 1.0 mM PPD with 0.2% (v/v) Tween-20 for 1.5 h and assayed in triplicate for $^{14}CO_2$ formation. Tissue was extracted and extracts analyzed by the organic acid and organic base columns. The experiment was performed similarly for [2,7-^{14}C]ALN except that 100 mg of tissue was incubated in 600 μl buffer.

Products	[2,7-^{14}C]ALN	[4,5-^{14}C]ALN
	μmol·g^{-1} fresh wt·h^{-1}	
Allantoate	3.0 ± 0.6	3.0 ± 0.6
CO_2	0.26 ± 0.01	0.12 ± 0.01
Glyoxylate	≤0.005	0.020 ± 0.004
Urea[a]	≤0.005	≤0.005
Glycine	≤0.005	0.010 ± 0.002
Serine[b]	≤0.005	0.025 ± 0.002
Total product[c]	0.13 ± 0.005	0.13 ± 0.01

[a] Urea was not detected either in the presence or absence of PPD. [b] Calculated assuming all three carbons are equally labeled. [c] Total product (excluding allantoate) expressed as μmol ALN catabolized/h·g fresh wt.

the organic acid and organic base columns. The potential products, ureidoglycolate, oxalurate, and urea (IPPD) were not present at the detection limit of 5 nmol·g^{-1} fresh wt·h^{-1}. These results are consistent with allantoate amidohydrolase action with a second step that similarly hydrolyzes the second ureido group.

Labeled Products of [4,5-^{14}C]Allantoin Metabolism. Leaf discs were incubated with [4,5-^{14}C]ALN from 0 to 90 min and extracted for analysis on the organic acid HPLC column. Although the specific activity was lower (*cf.* Figs. 5 and 6), the volume was minimized so that more label could be injected onto the HPLC column. The first product, [^{14}C]allantoate, was found in all samples from 10 to 90 min. Preliminary experiments established that unlabeled carrier glyoxylate was necessary for the recovery of [^{14}C] glyoxylate. Glyoxylate (Table III) was present at 0.03 μmol·g^{-1} fresh weight after 1.5 h of incubation. The presence of glyoxylate indicates that intermediates (ureidoglycolate or ureidoglycine) were not oxidized or reduced; for example, if oxalurate were an intermediate, oxamate or oxalate would be the product. No other labeled products were detected on the organic acid column.

Analysis of the organic base column effluent revealed that all of the apparent radiolabeled products, other than allantoate and glyoxylate, were in fractions OB8 to OB10, representing components that migrate from 8.4 to 9.6 min (Table II). To establish the exact migration of the labeled compound(s), 0.3 min fractions were collected instead of 1.0 min fractions. The label peaked in fraction 29 coincident with the A_{210} of unlabeled serine. The labeling pattern is consistent with serine being greater than 70% of the labeled product detected on the organic base column after a 20 min incubation. Serine production (Fig. 6) was approximately linear from 10 to 90 min and was present at a ratio (cpm) of 2:3 (CO_2:serine).

Identification of Labeled Fractions. Since comigration of OB10 with serine on one chromatography system is not definitive identification, further criteria were employed to identify the putative [^{14}C]serine derived from [4,5-^{14}C]ALN.

Fraction OB10 was dried under vacuum, redissolved in 10 μl of 1% (w/v) serine. Aliquots of 0.5 μl were applied to silica or cellulose TLC plates (3 μl total) and developed as described in "Materials and Methods." After a 2 week exposure, the spots visualized by autoradiography were exactly superimposable with ninhydrin-positive spots supporting the conclusion that OB10 was serine.

Carbamyl amino acids can be resolved by the organic acid column providing a simple second dimensional identification of serine and the other labeled compound(s) in OB8 to 10 (Table IV). Fractions OB8 to 10 were carbamylated with KCNO (16). The samples were dried under vacuum and injected onto the organic acid column. Table V outlines the results of this analysis. The majority of recovered label was carbamyl serine (52%), with lesser amounts of carbamyl glycine (26%) and carbamyl alanine (tentative identification) (14%). Injections prior to carbamylation showed no label in the carbamyl serine, carbamyl glycine, and carbamyl alanine fractions. An unknown peak (8% of recovered label) migrating at approximately 2.3 cm ± 0.1 cm, was in insufficient quantity to carry out its identification.

These data indicate that most of the labeled product from [4,5-^{14}C]ALN accumulated as [^{14}C]glyoxylate, [^{14}C]glycine, [^{14}C] serine, and $^{14}CO_2$, with no accumulation of ureido-intermediates at levels above the detection limit of 1 to 5 nmol·g^{-1} fresh weight·h^{-1}.

DISCUSSION

Our report is the first to indicate that urea is not involved in the primary catabolism of allantoin in soybean plants. There are two possible reasons why earlier workers were not previously led to this conclusion. Either the urea accumulation reported by others was nonenzymic or alternatively, there are two pathways of allantoate catabolism in plants.

Atkins *et al.* (1) found [^{14}C]urea in cowpea leaf tissue fed [2-^{14}C]ALN and in aphids feeding on this tissue. Their report does not, however, eliminate the possibility that urea is a product of aphid metabolism. In addition, their extracts from leaves were prepared using Dowex 50W (H^+), which under the acidic conditions employed could hydrolyze allantoate to urea and glyoxylate, as allantoate is unstable in acid (24).

The report of Shelp and Ireland (14) suggests that the AHA inhibition of $^{14}CO_2$ release from [2-^{14}C]ALN results from AHA inhibition of urease. However, we have found AHA to be a nonspecific inhibitor, inhibiting ALAH (27), allantoinase and

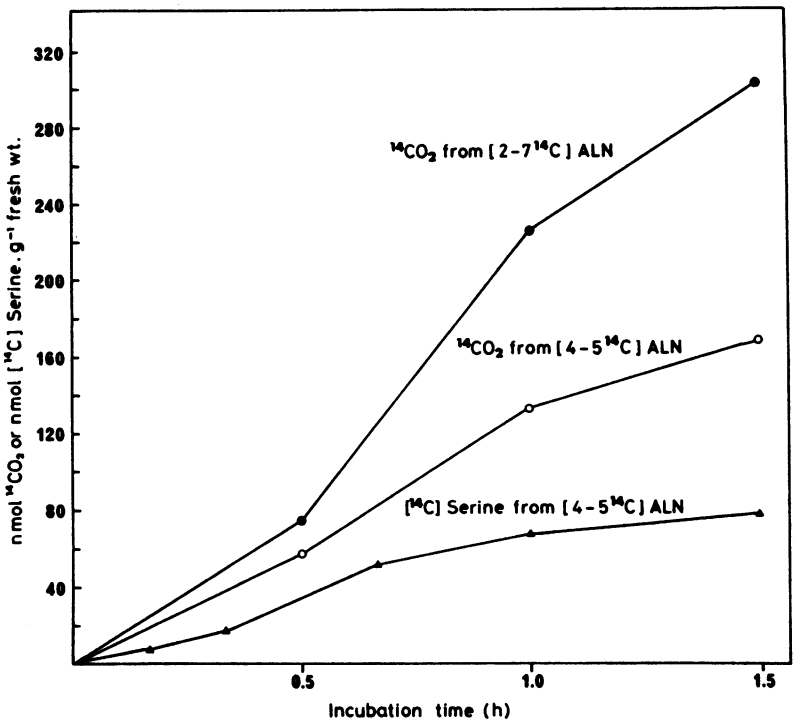

FIG. 6. Comparison of [^{14}C]serine and $^{14}CO_2$ release from [4,5-^{14}C]ALN and $^{14}CO_2$ release from [2,7-^{14}C]ALN. Leaf discs (50 or 100 mg) (single simples) were incubated with 1.3 mM [4,5-^{14}C]ALN or 1.3 mM [2,7-^{14}C]ALN and $^{14}CO_2$ was trapped on glass fiber filter discs impregnated with 150 μl 25% NaOH. Serine (▲) was quantitated by analysis on the organic base column. $^{14}CO_2$ was determined as described for Figure 3. $^{14}CO_2$ (●) from [2,7-^{14}C]ALN $^{14}CO_2$ (○) from [4,5-^{14}C]ALN.

Table IV. *HPLC Retention Times of Carbamyl Amino Acids on Aminex HPX-87A*

Carbamyl amino acids were synthesized by the procedure of Stark (16) and confirmed by commercial carbamyl serine and carbamyl glycine. Conditions for the organic acid column are the same as Table II.

Carbamyl Amino Acid	Organic Acid Migration
	min
Carbamyl serine	17.4
Carbamyl glycine (hydantoic acid)	26.0
Carbamyl alanine	30.2
Carbamyl glutamine	28.4
Carbamyl glutamate	21.4
Carbamyl asparagine	19.2

Table V. *Second Dimensional Separation of Fractions 8 to 10 of Organic Base Column*

An aliquot of an extract prepared from a 1 h [4,5-^{14}C]ALN incubation was chromatographed on the organic base column and 1 ml fractions were collected, dried under vacuum, redissolved in 100 μl H_2O and carbamylated ("Materials and Methods"). The carbamylated compounds were fractionated on the organic acid column and counted by liquid scintillation spectrometry.

Carbamyl Amino Acid	OB10	OB9	OB8
cpm in original fraction[a]	6,140	2,780	1,030
Carbamyl serine	80%	11%	0%
Carbamyl glycine	16%	47%	25%
Carbamyl alanine	0%	28%	58%
Unknown	4%	14%	17%
Recovery	90%	83%	75%

[a] In fraction OB8 to OB10.

glyoxylate reductase (6). The most critical data in the report of Shelp and Ireland (14), an accumulation of unlabeled urea in AHA-treated leaf slices fed ALN, was in part caused by loss of urea in the minus-AHA control rather than an allantoin-dependent increase in urea. Additionally, it is not clear that minus-ALN controls were included in their studies, leaving open the possibility that any urea buildup was not ALN-dependent (*e.g.* urea can be released from arginine by arginase).

Polacco *et al.* (11) found ALN-dependent NH_4^+ and urea production in crude extracts of developing soybeans, but they reported that the nonenzymic breakdown of an intermediate, (*e.g.* ureidoglycolate) could not be eliminated as a possible source of urea since incubations were greater than 10 h. Furthermore, PPD only partially blocked NH_4^+ release from allantoate. Ureidoglycolate is unstable under the reaction conditions employed.

Proposed Catabolic Pathway. Our data provide several lines of evidence establishing that in leaf discs allantoin is rapidly converted, via allantoinase, to allantoate which is further metabolized at a slower rate, to $4NH_4^+$, $2CO_2$, and glyoxylate. The second enzyme of the pathway is allantoate amidohydrolase, which hydrolyzes allantoate to NH_4^+, CO_2, and ureidoglycine or ureidoglycolate. This conclusion is based on the lack of urea accumulation (±PPD) from [2,7-^{14}C]ALN and on the lack of PPD inhibition of $^{14}CO_2$ release from [2,7-^{14}C]ALN. PPD has a K_i (*in vitro*) for soybean leaf urease of less than 20 μM (5), and inhibits leaf disc urease >99.9% under our conditions. Indirect evidence for a ureido-intermediate (probably ureidoglycine) other than ureidoglycolate was reported earlier (27). A second activity(ies) hydrolyzes ureidoglycolate or ureidoglycine to 2 or 3 NH_4^+, CO_2, and glyoxylate. As before, this conclusion is based on the finding that there is no urea accumulation in the presence of PPD and the failure of PPD to inhibit $^{14}CO_2$ release from [2,7-^{14}C]allantoin. These data agree with the conclusions of Polacco *et al.* (12) that established that ALN is at least partially degraded by soybean callus in the absence of urease, and the report of Winkler *et al.* (27) that proposed allantoate amidohydrolase to be the allantoate degrading activity of developing soybeans. The accumulation of [^{14}C]glyoxylate, [^{14}C]glycine, [^{14}C]serine, and $^{14}CO_2$ from [4,5-^{14}C]ALN unambiguously establishes that the metabolic pathway of allantoate metabolism is complete under our experimental conditions, *i.e.* that all C-N bonds are broken. The only pathway illustrated in Figure 1 that is consistent with our data is one in which all C-N bonds are broken independently of urease. We propose that allantoin is catabolized by a series of reactions outlined in Figure 7, including

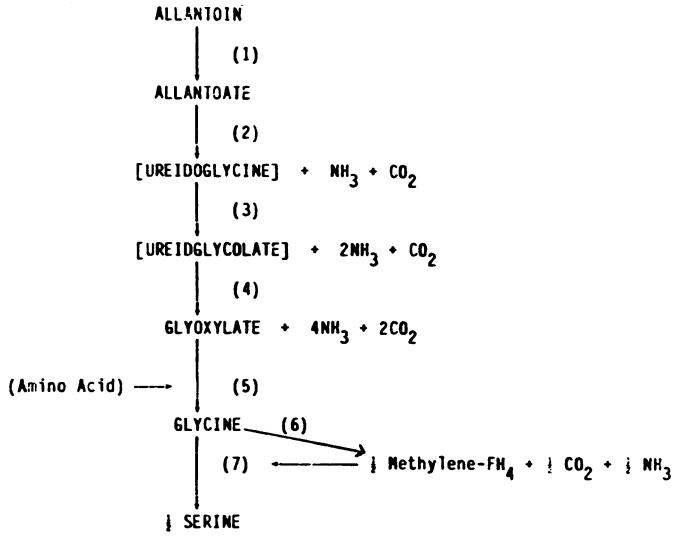

FIG. 7. Proposed pathway of ureide catabolism in soybean plants. (1), Allantoinase; (2), allantoate amidohydrolase; (3), ureidoglycine aminohydrolase; (4), ureidoglycolate amidohydrolase; (5), aminotransferase; (6), glycine decarboxylase; (7), serine hydroxymethyltransferase.

allantoin amidohydrolase, allantoate amidohydrolase, ureidoglycine aminohydrolase, and ureidoglycolate amidohydrolase. Allantoate amidohydrolase and ureidoglycine aminohydrolase may be activities of the same enzyme is proposed by Van der Drift *et al.* (22). The exact nature of the intermediates is at present uncertain because they are apparently below the detection limit of our analysis, but we have preliminary evidence for the presence of ureidoglycine and for a Mn^{2+}-dependent ureidoglycolate amidohydrolase in soybean seed coat extracts (RG Winkler, unpublished data).

The theoretical CO_2:serine ratio (cpm) from [4,5-^{14}C]ALN is 1:3 if glyoxylate is metabolized totally via the photorespiratory cycle; however, the observed ratio is 2:3 indicating that the 4 and 5 carbons may be oxidized to CO_2 *other* than via glycine decarboxylase. Additionally, the expected rate of [2,7-^{14}C]ALN-dependent $^{14}CO_2$ evolution *versus* that of [4,5^{14}C]ALN would be 4:1, but the observed was 4:1.7 (Fig. 5). It is possible that [^{14}C] glycine from [^{14}C]glyoxylate was preferentially decarboxylated in relation to unlabeled endogenous glycine, or that some [^{14}C] glyoxylate was oxidized directly by H_2O_2 (4) to release formate and CO_2. Since the leaf disc system was perturbed, *i.e.* exposed to high levels (nonphysiological) of glyoxylate, it is possible that a second metabolic pathway may have degraded glyoxylate. Glyoxylate is toxic to the cell, and does not accumulate in normal tissue. Its accumulation under our experimental conditions may flood the normal system of catabolism and allow for secondary reactions to occur that are not significant at "normal" levels of glyoxylate. Somerville and Ogren (15) have established that glycine decarboxylation is the sole site of photorespiratory CO_2 release under normal physiological conditions in *Arabidopsis*.

CONCLUSIONS

This study constitutes the first demonstration of the complete metabolism of ALN in a higher plant. We conclude that allantoate is metabolized by allantoate amidohydrolase and that the subsequent metabolism is most consistent with a second amidohydrolase (not urease) reaction that releases NH_4^+ and CO_2 from a ureido-intermediate which is either ureidoglycolate or ureidoglycine. Analysis of the products proves that all C-N bonds are broken under our experimental conditions, in agreement with the data of Coker and Schaeffer (2). The net products of this reaction are $4NH_4^+$, $2CO_2$, and 1 glyoxylate. Glyoxylate is metabolized by the photorespiratory cycle to give glycine, serine, and CO_2. We find no evidence that urea is a significant intermediate under our experimental conditions.

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