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Urease Is Not Essential for Ureide Degradation in Soybean[1]

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The hypothesis that soybean (*Glycine max* L. [Merrill]) catabolizes ureides to urea to a physiologically significant extent was tested and rejected. Urease-negative (*eu3-e1/eu3-e1*) plants were supported by fixed N_2 or by 2 mM NH_4NO_3, so that xylem-borne nitrogen contained predominantly ureides (allantoin and allantoic acid) or amide amino acids, respectively. Seed nitrogen yield was equal on either nitrogen regime, although 35-d-old fixing plants accumulated about 6 times more leaf urea. In callus, lack of an active urease reduced growth on either arginine or allantoin as the sole nitrogen source, but the reduction was greater on arginine (73%) than on allantoin (39%). Furthermore, urease-negative cells accumulated 17 times more urea than urease-positive cells on arginine; for allantoin the ratio was 1.8. Urease-negative callus accumulated urea at 3% the rate of seedlings. To test whether urea accumulating in urease-negative seedlings was derived from ureides, seeds were first allowed to imbibe in 1 mM allopurinol, an inhibitor of ureide formation. Seedling ureides were decreased by 90%, but urea levels were unchanged. Thus, ureides are poor precursors of urea, which was confirmed in seedlings that converted no more than 5% of seed-absorbed [^{14}C-ureido]allantoate to [^{14}C]urea, whereas 40 to 70% of [^{14}C-guanido]arginine was recovered as [^{14}C]urea.

The ureide allantoin and its derivative ureides, allantoate and ureidoglycolate, are purine breakdown products found throughout the plant kingdom (Schubert and Boland, 1990). In soybean (*Glycine max* L. [Merrill]), ureides are found in relatively high concentrations in seedlings (Fujihara and Yamaguchi, 1978; Polayes and Schubert, 1984). In addition, ureides are the major transport form of biologically fixed N_2 in soybean and in many other warm-weather legumes (Reynolds et al., 1982; Schubert and Boland, 1990). The ureide catabolic pathways of soybean and other plants are not fully understood, in contrast to those of microorganisms. Several bacteria (Vogels and van der Drift, 1976) and yeast (Cooper et al., 1979) convert allantoate to 2 urea + glyoxylate by the combined action of allantoate and ureidoglycolate amidinohydrolases, so that a block in urea assimilation results in the inability to utilize ureide N.

At least two reports indicate that ureide amidinohydrolases are operative in soybean. The xanthine dehydrogenase inhibitor AP (Triplett et al., 1980; Boland, 1981) blocked the accumulation of both ureides and urea in soybean seedlings 3 DAG (Fujihara and Yamaguchi, 1978). Acetohydroxamate, used as a urease inhibitor, stimulated accumulation of urea in leaf pieces floated on allantoin (Shelp and Ireland, 1985).

However, there is evidence that soybean contains ureide amidohydrolase activities, which release NH_3 and CO_2 directly from the ureido groups of allantoate and its derivative, ureidoglycolate (reviewed by Winkler et al., 1988). When synthesis of active urease in callus was inhibited by complexation of available nickel, urea-supported growth was blocked, but growth on allantoin N was not (Polacco et al., 1982). The urease inhibitor phenylphosphorodiamidate failed to block growth or to stimulate urea accumulation in soybean cell suspensions supported by ureide N (Stahlhut and Widholm, 1989). An allantoate amidohydrolase activity was characterized in extracts of immature soybean embryos and seed coats (Winkler et al., 1985). Subsequently, it was shown in intact soybean leaf tissue that both ureido groups of allantoate were converted to NH_3 and CO_2, even in the presence of phenylphosphorodiamidate (Winkler et al., 1987).

Since it is possible that soybean degrades ureides by both the amidohydrolase and amidinohydrolase pathways, we sought to determine whether amidinohydrolase catabolism of ureides by way of urea was physiologically significant throughout the life cycle of soybean. Significance was determined by seed protein yield in N_2-supported mutant plants unable to hydrolyze urea. We used the pleiotropic *eu2/eu2* and *eu3-e1/eu3-e1* mutants; each lacks the activities of both the embryo-specific and tissue-ubiquitous soybean urease isozymes (Meyer-Bothling et al., 1987). (Each isozyme is encoded by separate structural genes, unlinked to each other, to *Eu2*, or to *Eu3* [Torisky et al., 1994].) The absence of urease in *eu2/eu2* and *eu3-e1/eu3-e1* extends to at least one class of phylloplane bacteria, which are urease negative while inhabiting the mutant plants (Holland and Polacco, 1992).

The *eu2/eu2* and *eu3-e1/eu3-e1* plants accumulate urea (Stebbins et al., 1991). We tested ureides and Arg as potential urea precursors by comparing allantoin and Arg as the sole N source in urease-positive and -negative callus. The contributions of ureides and Arg to the seedling urea pool

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Abbreviations: AP, allopurinol; DAG, days after germination; R3, callus medium.

were determined by the generation of [^{14}C]urea in seedlings allowed to imbibe labeled allantoate and Arg.

Our results indicate that a portion of the ureides is catabolized to urea but that this pathway has little relevance for the growth and yield of soybean utilizing ureides as the sole or major N source.

MATERIALS AND METHODS

Plant Material

Whole Plants

The *eu2/eu2* and *eu3-e1/eu3-e1* mutants identify distinct loci; each mutant lacks the activity of both soybean-encoded (Torisky et al., 1994) urease isozymes, the ubiquitous and the embryo specific (Meyer-Bothling et al., 1987). In addition, at least one type of phylloplane bacteria is urease negative when associated with these mutants (Holland and Polacco, 1992). Segregating *eu3-e1/eu3-e1*, *Eu3/eu3-e1*, and *Eu3/Eu3* individuals in the F_5 or F_6 generation were from a second outcross of the original *eu3-e1/eu3-e1* isolate to cv Williams 82 (a derivative of the progenitor genotype Williams). Both *eu3-e1/eu3-e1* and *eu2/eu2* (similarly backcrossed) were used in callus growth studies. In seedling metabolite accumulation studies, seeds were always surface sterilized (95% ethanol for 2–3 min; 10% commercial bleach, 0.1% [w/v] SDS for 3 min; several washes in sterile deionized water) and germinated at 28°C in rolls of autoclaved germination paper (Anchor Paper Co., St. Paul, MN) saturated with sterile water. Urease-positive seedlings were distinguished from urease-negative siblings by a "cotyledon chip" assay (Meyer-Bothling et al., 1987). To determine AP effects, seedlings were germinated in rolls of germination paper maintained upright in a solution of 1 mM AP or in water until 8 d after imbibition (7 DAG). AP had no effect on germination rate or on radicle length. For hydroponic studies, nonsterile seedlings were transplanted to perlite:sand (approximately 1:1 [v/v]); roots were continuously bathed by bottom irrigation in a complete nutrient solution (Triplett et al., 1981) containing 1 μM $NiSO_4$. N was either 2 mM NH_4NO_3 or fixed N_2 in plants inoculated with *Bradyrhizobium japonicum* (U.S. Department of Agriculture strain 110). Plants were sown in mid-April and maintained in a greenhouse under natural light until seed harvest in early October.

Callus

Callus was induced from expanding leaves and maintained on R3 medium (Polacco, 1976). For growth determinations callus was maintained for 26 d on the respective N sources, including plates lacking N. Standard transfers (approximately 10 mg dry weight = five callus pieces/60-mm plate) were made to fresh plates and dry weight was determined by lyophilization after 27 (Williams 82) and 33 d (*eu2/eu2* and *eu3/eu3*). Two to three plates were used per genotype-N source combination, and milligram dry weight per plate, in excess of dry weight on minus-N, was determined. Callus growth on urea, allantoin, or Arg was normalized to growth on NH_4Cl.

For urea accumulation studies callus was transferred to modified R3 (total N, as KNO_3/NH_4NO_3, reduced from 60 to 40 meq L^{-1}) for 15 to 30 d and then transferred to N-free R3 for another 8 to 13 d to deplete nitrogenous reserves. Replicate cultures were supplemented with either 10 mM K citrate (pH 6.0) or 10 mM K citrate (pH 6.0) plus 10 μM $NiSO_4$. Citrate (10 mM) induces a urease-negative phenocopy by complexing trace nickel, whereas added 10 μM $NiSO_4$ provides enough nontoxic free nickel to stimulate maximal growth with urea as the N source (Polacco, 1977). Callus was then transferred from N-free medium to media containing KNO_3/NH_4NO_3, allantoin, or Arg (all at 40 meq N L^{-1}). After 7 d callus was harvested, blotted dry, frozen, and lyophilized, prior to extraction.

Metabolite and Urease Determinations

Tissue extraction and urea analysis were as previously described (Stebbins et al., 1991). Ureides (allantoin and allantoate) were measured according to the method of Vogels and van der Drift (1970). Arg determinations were based on the procedures of Lovatt and Cheng (1984), except that, instead of determining $^{14}CO_2$ derived from combined arginase/urease action on metabolites that had incorporated $^{14}CO_2$, we determined NH_4^+ colorimetrically (Stebbins et al., 1991) from duplicate samples treated either with arginase/urease or with urease alone. The difference was taken to be NH_4^+ generated from the Arg guanido moiety.

Seed was quantitatively harvested from hydroponically grown greenhouse plants and maintained over dry $CaCl_2$ at 4°C. Weighed portions (generally 5 g of the 15- to 20-g seed yield) were ground in a coffee grinder. Separate 1-g batches were used to determine dry weight (drying to constant weight at 50°C), urea, and total N. The latter was determined by total combustion and conversion to N_2 (Sweeney, 1989) in a combustion apparatus (Leco Corp, St. Joseph, MI) in the Missouri Agricultural Experiment Station Chemistry Laboratories.

Urease was determined in whole lyophilized callus essentially as described by Polacco et al. (1989) and was expressed as nmol urea hydrolyzed h^{-1} mg^{-1} dry weight.

[Ureido-^{14}C]Allantoate and [Guanido-^{14}C]Arg Labeling of Seedlings

L-[guanido-^{14}C]Arg (55.6 mCi/mmol, New England Nuclear) and [ureido-^{14}C]allantoate (potassium salt, 1.45 mCi/mmol [Winkler et al., 1987]) stocks were lyophilized, rehydrated with H_2O, and filter sterilized. Solutions for seed imbibition were diluted to 0.98 and 0.81 mCi mL^{-1}, respectively, for Arg and allantoin label.

Surface-sterilized seeds were air dried and placed in a Petri dish between glass-fiber discs (2.1 cm diameter) to which labeled precursor (approximately 500 μL/seed) was applied. Seeds were allowed to imbibe for 15 h and then rinsed; excess liquid was removed, and then seeds were germinated in sterile germination paper rolls as described above. Seedlings were harvested after 4 d (3 DAG), separated into radicles and cotyledons, frozen, and lyophilized.

Radicles and cotyledons were ground in cold 3% (v/v) perchloric acid (Stebbins et al., 1991) and neutralized with approximately 3 volumes of 2.5 M K_2CO_3 and 1 mM EDTA. Cleared cotyledon extracts were defatted by petroleum ether extraction, lyophilized, and stored at −80°C until analysis.

[^{14}C]Urea derived from L-[guanido-^{14}C]Arg or [ureido-^{14}C]allantoate was determined by urease (Sigma, fraction IX) treatment and trapping $^{14}CO_2$ (Torisky and Polacco, 1990). To a portion of each extract was added: 3 to 10 μL of 10 N HCl (until the solution became neutral as determined by cresol red [5 μL of 100 mg mL^{-1} solution 0.1 mL^{-1} extract] turning from red to barely pink); 10 μL of 1 M potassium phosphate buffer, pH 7.0; and water to approximately 1 mL total volume. After the samples were split into two aliquots, 15 μL of urease (Sigma type IX, 10 mg mL^{-1} in 50 mM potassium phosphate buffer, 5 mM DTT, pH 7.2) were added to one, and both were incubated for 30 min at 37°C. The difference in $^{14}CO_2$ release was equated to the total amount of urea in the original extract.

RESULTS

Effect of N Source on Yield and Urea Accumulation in Urease-Negative Soybean

We tested the hypothesis that ureides are catabolized, wholly or in major part, to urea (the amidinohydrolase pathway). This hypothesis predicts that blocking urea catabolism will severely reduce plant seed yield and callus growth when ureides are the major or sole N source, respectively.

Plants

Urease-negative and urease-positive sibling plants were grown hydroponically with N provided either as NH_4NO_3 or from biological fixation. The two phenotypes (urease-negative *eu3-e1/eu3-e1* versus urease positive *Eu3/eu3-e1* and *Eu3/Eu3*) are otherwise nearly isogenic, since they were generated from five rounds of self-pollination from a single heterozygous seed. The lack of urease did not significantly reduce leaf N (expressed as a percentage of leaf dry weight) under N_2 fixation conditions (Fig. 1). In fact, the quotient leaf N in N_2-fixing divided by leaf N in NH_4NO_3-fed plants was higher in urease-negative than in urease-positive plants. Thus, the data concerning leaf N do not support the hypothesis that ureides are catabolized wholly or in major part to urea.

Also inconsistent with the hypothesis was that only one-fifteenth of leaf N was in urea in N_2-fixing *eu3-e1/eu3-e1* plants, although this figure represented about 6 times the leaf urea accumulated in NH_4NO_3-supported *eu3-e1/eu3-e1* plants (Fig. 1). Urea was barely detectable in leaves of urease-positive siblings grown under both N regimes. We point out that the metabolic origin of leaf urea in *eu3-e1/eu3-e1* plants was not established. However, leaves from N_2-fixing plants (35 DAG) of both urease phenotypes had ureide pools 5 to 8 times larger than those of NH_4NO_3-fed plants (45 to 6 μmol g^{-1} dry weight for *eu3-e1/eu3-e1* and 20 to 4 for urease-positive siblings).

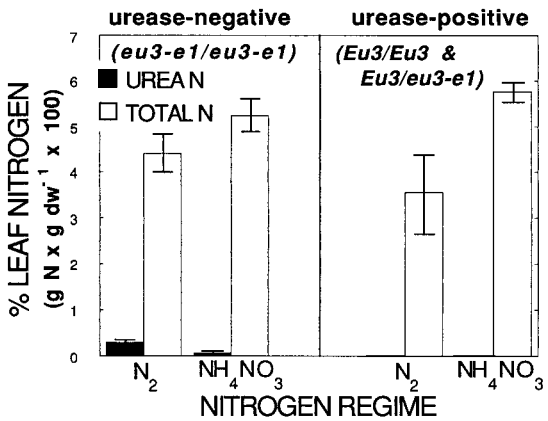

Figure 1. Influence of biological N_2 fixation on urea accumulation and N content of trifoliate leaves of urease-positive (*Eu3/Eu3* and *Eu3/eu3-e1*) and urease-negative (*eu3-e1/eu3-e1*) sibling plants. Urea and total N were determined separately on portions of lyophilized trifoliate leaves of plants 35 DAG. (Leaves were pooled, since there was no clear pattern in urea levels among leaves from the plant top or bottom.) Urease-negative (*eu3-e1/eu3-e1*) and urease-positive (*Eu3/[]*) plants were siblings in the F_5 generation of single-seed descent from an *Eu3/eu3-e1* individual. Results are means ± SD; *n* = three to five plants. dw, Dry weight.

The most stringent test of the hypothesis is seed protein yield; stoichiometric or major conversion of ureido moieties to urea will severely reduce protein deposition in N_2-fixing *eu3-e1/eu3-e1* plants unable to assimilate urea. Since urease-positive and urease-negative siblings did not differ dramatically in plant appearance or in leaf N content (Fig. 1), we concentrated on the effect of the N regime on seed protein yield within the *eu3-e1/eu3-e1* genotype. Hydroponic plants supported by biological N_2 fixation or by NH_4NO_3 were grown to maturity. Only fixing plants exhibited root nodules. Plants of both N regimes exhibited robust growth. More importantly, N_2-supported plants yielded at least as much seed protein (percentage of seed N × total seed dry weight × 6.25) as plants supported by NH_4NO_3 (7.2 ± 1.5 versus 5.9 ± 2 g, respectively; Fig. 2). The calculated crude seed protein content (percentage of seed N × 6.25) of N_2-supported *eu3-e1/eu3-e1* was 38.6% and compares relatively well with a value of 41.3% reported in multisite field trials of Williams 82 (Wilcox, 1985).

The small portion of total seed N that was urea-N (1.1 and 2% in seeds from NH_4NO_3- and N_2-supported plants, respectively [Fig. 2]) is further evidence against a significant conversion of ureides to urea. An earlier report indicated that the urea that accumulated in urease-negative seeds was delivered from maternal sources (Stebbins et al., 1991). The relatively higher pools of leaf (Fig. 1) versus seed urea (Fig. 2) in N_2-fixing *eu3-e1/eu3-e1* plants suggest that leaf urea is not delivered to the developing pod as efficiently as other nitrogenous compounds.

Callus

We compared the growth of urease-negative (*eu2/eu2* and *eu3-e1/eu3-e1*) and urease-positive ("wild type" = pro-

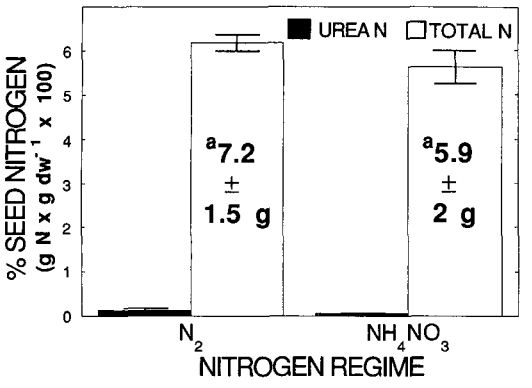

Figure 2. Influence of biological N_2 fixation on seed urea accumulation, seed N content, and total crude seed protein yield of urease-negative (*eu3-e1/eu3-e1*) plants. Urea and total N were determined separately on portions of pooled and ground mature seeds quantitatively harvested from NH_4NO_3- and N_2-supported *eu3-e1/eu3-e1* plants (n = 8 and 10 pooled seed lots, respectively). [a]Crude seed protein was determined from the percentage of seed N (Wilcox, 1985): percentage of seed N × total seed dry weight (dw) × 6.25.

genitor genotype Williams 82) callus on four N sources (all at 10 meq N L^{-1}): NH_4Cl, urea, allantoin, or Arg. These N sources resulted in different dry weight yields of urease-positive callus; urea supported 50% more callus growth than did NH_4Cl. As expected, urease-negative callus yields were zero on urea-N, but, significantly, they were greater on allantoin than on NH_4Cl (Table I). It is pointed out that urease-negative callus yields on allantoin and Arg were both reduced relative to wild-type callus. However, the reduction was greater for callus cultured on Arg (73%) than on allantoin (39%).

These callus yield patterns of Table I indicate that only a minor portion of allantoin is degraded to urea and that Arg may be a more significant generator of urea. In agreement, urease-negative callus (*eu3-e1/eu3-e1* or *Eu3/Eu3* made into a urease-negative phenocopy by Ni deprivation [Polacco, 1977]) consistently accumulated more urea when cultured on Arg than callus cultured on allantoin (Table II). Whereas allantoin-utilizing cells accumulated urea, the ratio of accumulated urea in urease-negative versus urease-positive callus was 1.8 for allantoin (3.4 ÷ 1.9) and 17 for Arg (8.5 ÷ 0.5). Although Arg appears to be the more effective urea precursor, we cannot exclude the possibility that ureides are metabolized to urea at 10 to 20% the rate of Arg conversion. (A possible explanation for the relatively high urea level in allantoin-supported urease-positive callus is nonenzymatic breakdown in the culture medium during the 7-d culture. Our extraction and analytical procedures do not generate urea from ureides, since leaves from N_2-fixing, urease-positive plants exhibited high ureide and essentially zero urea pools [Fig. 1].)

Since little or no urea accumulated in KNO_3/NH_4NO_3-supported callus (approximately 0.16 μmol urea g^{-1} dry weight d^{-1}), urea is not an important product of intermediary callus metabolism. This suggests, but does not prove, that urea in allantoin- and Arg-supported callus was derived from exogenous allantoin and Arg, respectively. We therefore turned our attention to the seedling, a tissue that generates urea from endogenous reserves (approximately 6 μmol urea g^{-1} dry weight d^{-1}, data not shown), to determine metabolic origins of urea.

Table I. *Growth of soybean callus on NH_4Cl, urea, allantoin, or Arg as sole N source*

Genotype	N Source (10 meq L^{-1})	Relative Callus Yield[a]
Williams 82	NH_4Cl	1.0
(*Eu2/Eu2,Eu3/Eu3*)	Urea	1.5
	Allantoin	1.8
	Arg	3.0
eu2/eu2	NH_4Cl	1.0
	Urea	0
	Allantoin	1.1
	Arg	0.8
eu3-e1/eu3-e1	NH_4Cl	1.0
	Urea	0
	Allantoin	1.1
	Arg	0.8

[a] Callus yield per plate (mg dry weight − mg dry weight on N-free medium) was determined for each N source and divided by the value for the NH_4Cl N source (for which Williams 82, *eu2/eu2*, and *eu3-e1/eu3-e1* were 4, 13.3, and 20 mg/plate, respectively). Callus was maintained on respective N sources for about 3 weeks followed by standard transfers (approximately 10 mg dry weight = five callus pieces/60-mm plate) to fresh plates. Dry weight was determined by lyophilization after 27 (Williams 82) and 33 d (*eu2/eu2* and *eu3/eu3*). Two to three plates were used per genotype-N source combination.

Table II. *Accumulation of urea in soybean callus maintained 7 d[a] on NH_4/NO_3, allantoin, and Arg N sources*

Genotype	Ni^{2+}[b]	N Source (40 meq N L^{-1})	Urease[c]	Urea
	μM		μmol g^{-1} dry wt	
	10	KNO_3/NH_4NO_3	55	0.7
Eu3/Eu3	10	Allantoin	84	1.9
	10	Arg	78	0.5
	0	KNO_3/NH_4NO_3	0.20	1.1
Eu3/Eu3	0	Allantoin	0.15	3.4
	0	Arg	0.2	7.0
	0	KNO_3/NH_4NO_3	0.01	0
eu3-e1/eu3-e1	0	Allantoin	0.03	4.1
	0	Arg	0.02	6.6
	10	KNO_3/NH_4NO_3	0.01	0
eu3-e1/eu3-e1	10	Allantoin	0.02	2.7
	10	Arg	0.01	11.8

[a] Callus was maintained on N-free medium for 8 to 13 d to deplete nitrogenous reserves; it was then grown on the indicated N sources and harvested after 7 d. [b] Nickel was provided as $NiSO_4$ in the presence of 10 mM K citrate, pH 6.0. Citrate was also included in culture media lacking added nickel. It has been shown that addition of 10 mM K citrate to basal medium effectively deprives cells of trace nickel (Polacco, 1977). [c] Urease (nmol urea hydrolyzed h^{-1} mg^{-1} dry weight) was determined on a sample of the lyophilized callus used for urea determinations (Polacco et al., 1989).

Table III. *Nitrogenous metabolite accumulation in urease-positive and urease-negative seedling siblings 3 DAG*

Urease-positive and -negative seedlings were progeny of a selfed *Eu3/eu3-e1* plant (see legend, Fig. 1). Since the seeds developed on a urease-positive plant, they accumulated no urea prior to germination (Stebbins et al., 1991).

Genotype (phenotype)	Part of Seedling	Metabolite		
		Urea	Ureides	Arg
		μmol g⁻¹ dry wt		
Eu3/Eu3 and Eu3/eu3-e1 (urease positive)	Cotyledon	0.4 ± 0.3 (*n* = 6)	10 ± 2 (*n* = 4)	15 ± 7 (*n* = 7)
	Radicle	1 ± 1 (*n* = 4)	58 ± 4 (*n* = 4)	2 ± 1 (*n* = 4)
	Entire seedling[a]	0.5 [0.1]	16.2 [2.3]	13.3 [1.9]
eu3-e1/eu3-e1 (urease negative)	Cotyledon	10 ± 3 (*n* = 7)	13 ± 6 (*n* = 4)	15 ± 7 (*n* = 7)
	Radicle	20 ± 7 (*n* = 5)	42 ± 9 (*n* = 5)	5 ± 2 (*n* = 5)
	Entire seedling[a]	11.3 [1.6]	16.7 [2.4]	13.7 [2.0]

[a] Seedlings were separated into radicle and cotyledon sections upon which metabolite determinations were made. Values for entire seedlings were calculated from the data presented here and from seedling tissue weights. They were expressed in two ways: as μmol g^{-1} dry weight and as μmol/seedling (value in brackets) for direct comparison with values of Figure 3.

Urea Accumulation in Urease-Negative Seedlings Is Not Dependent on a Large Ureide Pool

Progeny seedlings (3 DAG) from a selfed heterozygous *Eu3/eu3-e1* plant were separated into urease-positive and urease-negative siblings. Since both urease-negative and urease-positive seeds developed on a urease-positive maternal plant, neither sibling had accumulated urea (Stebbins et al., 1991), allowing us to focus on urea that accumulated during germination on water.

Urease-negative seedlings accumulated >20 times more urea than urease-positive siblings in both the radicle and cotyledon (Table III). There was no major difference in the ureide or Arg pools, although the data do not allow us to assess their relative turnover in high and low urea backgrounds. In both urease phenotypes, ureides were more abundant than Arg in the radicle, whereas Arg was more abundant in the cotyledon.

To determine whether urea accumulating in *eu3-e1/eu3-e1* seedlings was derived from ureides or Arg, we first sought to block the accumulation of ureides by inhibiting purine oxidation. We used AP, which has been demonstrated to inhibit xanthine dehydrogenase in nodules of soybean (Triplett et al., 1980) and navy bean (Boland, 1981). There was little or no inhibition of germination or radicle elongation by 1 mM AP. AP application, however, resulted in ≥90% reduction of seedling (7 DAG) ureide pools with little or no decrease in accumulated urea (Fig. 3). The data in Figure 3 were combined from separate determinations on radicle and cotyledon.

More [^{14}C]Urea Is Generated from [Guanido-^{14}C]Arg than from [Ureido-^{14}C]Allantoate in 3 DAG Seedlings

To confirm that seedling urea had a nonureide source, we compared Arg and allantoate as labeled precursors of [^{14}C]urea. *eu3-e1/eu3-e1* seedlings were allowed to imbibe in Arg and the potassium salt of allantoic acid, compounds labeled exclusively in the moieties released as urea from arginase action (the guanido group of Arg) or from amidinohydrolase action (the ureido groups of allantoate). Liberated urea was determined in seedling extracts by the urease-catalyzed release of $^{14}CO_2$. Although we cannot determine how accessible the in vivo pools were to the added labeled precursors, it is clear that Arg was effectively converted to urea, whereas allantoate was not: 40 and 70% of Arg compared to 2% of allantoate label (Table IV). At least 40% of [ureido-^{14}C]allantoate taken up was extensively metabolized in that it was not recoverable as allantoate or ureidoglycolate. Since any $^{14}CO_2$ evolved during the 0 to 3 DAG interval was not trapped, 40% is an underestimate of the extent of allantoate metabolism. Therefore, a maximum of 5% (2/40+%) of the degraded ureido moieties of allantoate was converted to urea.

DISCUSSION

In yeast, the ureide allantoin is catabolized exclusively to 2 urea + glyoxylate so that a block in urea assimilation results in a block in ureide assimilation (Cooper et al., 1979). However, soybean clearly does not assimilate allantoin by way of stoichiometric conversion to two molecules of urea, since inability to assimilate ureido-N should have

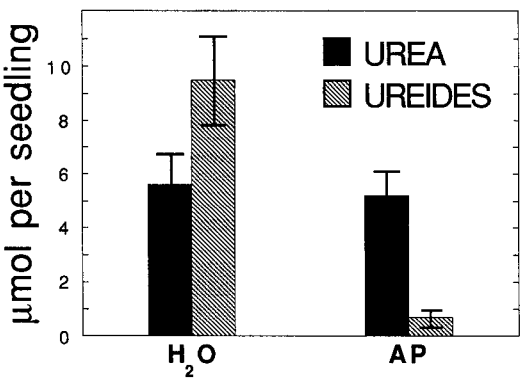

Figure 3. Effect of AP on accumulation of nitrogenous metabolites in urease-negative seedlings (*eu3-e1/eu3-e1*). Segregating progeny seed from a selfed *Eu3/eu3-e1* plant were germinated under sterile conditions in water or in 1 mM AP and maintained in germination paper rolls until 7 DAG. Urease-negative (*eu3-e1/eu3-e1*) seedlings were identified from each treatment by a cotyledon sliver urease assay. Means ± SD of three separate determinations for each treatment are shown.

Table IV. *Distribution of ^{14}C in eu3-e1/eu3-e1 3 DAG seedlings allowed to imbibe either [ureido-^{14}C]allantoate or L-[guanido-^{14}C]Arg*

Results are averages ± SD of three determinations.

Precursor	Percent ^{14}C Tissue Distribution[a]		Percent ^{14}C as [^{14}C]Urea[b]	
	Cotyledon	Radicle	Cotyledon	Radicle
[Ureido-^{14}C]-Allantoate	33 ± 15	67 ± 15	2 ± 1	2 ± 0.3
L-[Guanido-^{14}C]Arg	79 ± 3	21 ± 3	40 ± 13	70 ± 6

[a] A range of 1000 to 8000 cpm mg^{-1} dry weight (radicles) and 100 to 1000 cpm mg^{-1} dry weight (cotyledons) was recovered from extracts of three different washed seedlings allowed to imbibe in [ureido-^{14}C]allantoate. The values for seedlings allowed to imbibe in L-[guanido-^{14}C]Arg were 300 to 2000 (radicles) and 300 to 700 (cotyledons). [b] [^{14}C]Urea was determined from the urease-catalyzed release of $^{14}CO_2$, as described in "Materials and Methods."

significantly diminished the performance of N_2-supported urease-negative *eu3-e1/eu3-e1* plants. "Significance" in an agronomic sense for soybean is seed yield, generally seed protein yield, and this was approximately equivalent in urease-negative plants supported either by biologically fixed N_2 or by NH_4NO_3 fertilization. In the former, 70 to 90% of N transported from actively fixing nodules is in the form of the ureides allantoin and allantoate, whereas in the latter, amide amino acids are the major xylem form of N (Reynolds et al., 1982). Lack of stoichiometric conversion of ureido-N to urea is clearly demonstrated in callus, which showed only partial growth inhibition on allantoin-N when urea assimilation was completely blocked (by the *eu3-e1* or *eu2* lesions). Arg-supported callus generated 10 times as much urea as callus utilizing allantoin (Table II), a value derived from comparison of the ratio of urea generation in urease-negative versus urease-positive callus.

Although accumulation patterns in callus suggest that urea is derived from Arg and, to a lesser extent, from allantoin N sources (little or no urea accumulated on KNO_3/NH_4NO_3 N source [Table II]), we cannot formally assign a precursor(s) to unlabeled callus urea. We thus used labeled precursors in the seedling for two reasons: (a) the seedling is a more "natural" developmental stage than callus and (b) its intermediary metabolism does generate urea (Table III). Although seedlings are rich in ureides (Fujihara and Yamaguchi, 1978; Fig. 3), labeling studies suggested that seedling urea is generated from Arg and not from ureides. About 40% of seedling label from [^{14}C-guanido]Arg was recovered in [^{14}C]urea as compared to 2% from [^{14}C-ureido]allantoate (Table IV). In unlabeled seedlings 7 DAG, AP reduced the ureide pool by 90% but had little or no effect on accumulated urea (Fig. 3). Fujihara and Yamaguchi (1978), on the other hand, found that AP (1 mM) inhibited urea accumulation in the approximately 3-DAG seedling axis. We suggest that the low ureide:urea ratio that they reported in urease-positive seedling axes (1.33, compared to ours of approximately 60) indicates that urea was an artifact of allantoate decomposition.

The results reported here suggest that amidohydrolase activities (Winkler et al., 1985, 1987, 1988), which release NH_4^+ and CO_2 directly from allantoate and its product, ureidoglycolate, catalyze the major reactions for ureide assimilation in soybean. This is in agreement with previous studies in cultured soybean cells (Polacco et al., 1982; Stahlhut and Widholm, 1989). It is true that urea accumulation in leaves of N_2-fixing plants (Fig. 1) and in allantoin-utilizing callus (Table II) indicates degradation of a portion of ureides to urea, in agreement with our earlier observation of both ammonia and urea production from allantoate in developing seed extracts (Polacco et al., 1985). However, the ureide to urea (amidinohydrolase) pathway has little relevance for the growth and yield of N_2-fixing greenhouse-grown soybean.

Arg is a logical precursor of urea, especially in germinating seeds (Polacco and Holland, 1993). Arg accounts for up to 40% of mature seed N in many angiosperms (VanEtten et al., 1963); in soybean it contains 18% of protein-bound N (Micallef and Shelp, 1989a). The soybean 7S and 11S storage proteins contain more than 6 mol% Arg (Utsumi et al., 1987; Sebastiani et al., 1990), whereas Arg is present at 3 mol% in an "average" protein (VanEtten et al., 1967). Thus, during soybean germination there is a net breakdown of Arg as the amino acids released from storage proteins are reconfigured to the profile of "metabolically active proteins." Consistent with this assertion is the rapid increase in arginase (EC 3.5.3.1) activity in axes of germinating soybean (Kang and Cho, 1990) and the accumulation of urea in urease-negative seedlings (Stebbins et al., 1991).

Developing legume seeds are often rich in free Arg. For example, at least 50% of free amino acid N in developing pea (DeRuiter and Kollöffel, 1983) and soybean (Micallef and Shelp, 1989a) is in the Arg pool. However, in contrast to the arginase of soybean seedlings, the active arginase (Micallef and Shelp, 1989b; Kang and Cho, 1990) of developing seeds does not appear to degrade Arg in vivo: there is no urea accumulation in urease-negative *eu3-e1/eu3-e1* seeds developing on urease-positive (*Eu3/eu3-e1*) plants. Urease-negative embryos accumulate urea only when developed on urease-negative (and urea-accumulating) plants, suggesting that neither Arg nor any other compound generated urea within the developing embryo and that urea was delivered to the embryo from maternal tissue (Stebbins et al., 1991).

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