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Arginine Metabolism in Developing Soybean Cotyledons[1]

II. Biosynthesis

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ABSTRACT

Tracer kinetic experiments were performed using [ureido-^{14}C] citrulline, [1-^{14}C]ornithine, and isotope trapping techniques to determine if arginine is synthesized via the urea cycle in developing cotyledons of *Glycine max* (L.) Merrill. Excised cotyledons were injected with the ^{14}C-solution and incubated in sealed vials containing a CO_2 trap. The free and protein amino acids were analyzed using high performance liquid chromatography and arginine-specific enzyme-linked assays. In the ^{14}C-citrulline feeding experiment argininosuccinate was the most highly labeled compound after 5 minutes and it was the first compound to lose ^{14}C later in the time course. Carbon-14 was also recovered in free arginine, protein arginine, and CO_2 up to 4 hours after introduction of label. All of the ^{14}C in free and protein arginine could be accounted for in the C-6 position. Metabolism of ^{14}C-ornithine resulted in ^{14}C-incorporation into citrulline and free and protein arginine and in the evolution of $^{14}CO_2$. Citrulline was the most highly labeled compound after 15 minutes and was the first compound to reach a steady state level of ^{14}C. With the addition of 800 nanomoles unlabeled citrulline to the ^{14}C-ornithine feeding solution citrulline was the only compound labeled after 5 minutes and the steady state level of ^{14}C-citrulline increased 12-fold. The appearance of ^{14}C in free arginine and protein arginine was also delayed. In both ^{14}C-ornithine feedings all of the ^{14}C in free and protein arginine could be accounted for in the C-1 position. Together, the data support the reaction sequence: ornithine → citrulline → argininosuccinate → arginine → protein arginine.

Arginine functions as a nitrogen transport molecule (15) and as an important nitrogen storage form in plants. In seeds it can constitute up to 40% of the nitrogen in storage protein (20), which explains why seeds are used as the predominant source of material to study Arg metabolism in plants. Most work on Arg metabolism in plants has investigated the utilization of this amino acid (3, 19), but studies on its biosynthesis are lacking.

Evidence suggests that plants convert Glu into Orn via acetylated glutamate derivatives, and then Orn is converted into Arg through the following reaction sequence, Orn, Cit, ArgS, and Arg, also known as the urea cycle (5, 19). However, tracer studies have not satisfactorily elucidated either the reaction sequence in the conversion of Orn to Arg or the involvement of ArgS in Arg biosynthesis.

Jones and Boulter (10) fed [U-^{14}C]Arg to germinating *Vicia faba* seeds and recovered ^{14}C in ArgS. However, Pro was labeled first, followed by Orn and ArgS, and then Cit. This labeling pattern suggests that catabolism of Arg was occurring but does not support a role for ArgS in Arg biosynthesis. ArgS was also labeled when [ureido^{-14}C]Cit was applied to spruce buds (8). However, the tissue was not sampled until after 6 h when ^{14}C was also recovered in urea, Arg, guanidino compounds, and several unidentified compounds. Thus, the exact involvement of ArgS in Arg biosynthesis remains uncertain.

In this study, tracer kinetic experiments were performed using [ureido-^{14}C]Cit, [1-^{14}C]Orn, and isotope trapping techniques to determine if Arg is synthesized via the urea cycle in developing soybean cotyledons, which are believed to actively synthesize Arg (13).

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max* (L.) Merrill cv Maple Arrow) plants, effectively nodulated with *Bradyrhizobium japonicum* (The Nitragin Company, Milwaukee, WI) were grown in 9 L pots (6 plants per pot) containing vermiculite in a naturally lighted greenhouse as previously described (13).

Seeds were randomly selected from plants at 23 DAA,[2] the cotyledons were excised, and one cotyledon was retained from each seed. Care was taken to separate the embryo sac tissue from the cotyledons. Tissue was kept on ice during all steps.

Injection and Incubation Procedures

L-[Ureido-^{14}C]Cit (42.4 mCi mmol^{-1}), L-[1-^{14}C]Orn (54.3 mCi mmol^{-1}), and [^{14}C]urea (4.5 mCi mmol^{-1}) were obtained from New England Nuclear. The shipping medium was evaporated under a stream of ultrapure N_2 and the ^{14}C-compound was resuspended in 50 mM Hepes (pH 8.0). Four microliters of the ^{14}C-solution (7.5–10.3 × 10^5 dpm) was injected into an excised cotyledon, which was then incubated at 30°C in a 34 mL serum bottle containing a CO_2 trap as described by Micallef and Shelp (13).

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[2] Abbreviations: DAA, days after anthesis; ArgS, argininosuccinate; OPA, *o*-phthalaldehyde.

Free Amino Acid and Protein Extraction

Tissue was ground in sulfosalicylic acid (30 mg/mL), the supernatant was collected, and the protein was extracted and hydrolyzed in 6 N HCl (13).

Sample Analysis

The free amino acid supernatant was analyzed by reverse-phase HPLC following derivitization with OPA and the radioactivity quantified by fraction collection-liquid scintillation spectrometry as described by Micallef *et al.* (14). That study showed that OPA-amino acids were unstable on the column; however, it is possible to determine correction factors for the compounds of interest, provided that ^{14}C-label is present in only a limited number of amino acids. The correction factors for Glu, Cit, Arg, and Orn under those chromatographic conditions were 1.22, 1.56, 1.54, and 1.56, respectively. The ^{14}C quantified for ArgS may be an underestimate because a ^{14}C-standard was not available for determination of a correction factor.

The total radioactivity in the free amino acid supernatant and the protein hydrolysate was determined by liquid scintillation spectrometry (13).

Enzymic Analysis

The ^{14}C in the C-1 and C-6 positions of free and protein Arg was quantified respectively using an Arg decarboxylase assay described by Micallef and Shelp (13), and an arginase-urease assay developed by Lovatt and Cheng (12).

Statistical Analysis

Treatment of the data was conducted using the paired analysis procedure (18). Significance indicates significance at the 5% level.

RESULTS

Products of [Ureido-^{14}C]Citrulline Metabolism

Preliminary experiments using [ureido-^{14}C]Cit and [1-^{14}C]Orn demonstrated that ^{14}C-labeled urea cycle intermediates were detectable in soybean cotyledons 23 DAA (data not shown). The metabolism of [ureido-^{14}C]Cit by cotyledons resulted in the incorporation of ^{14}C into ArgS, free and protein Arg, and the release of $^{14}CO_2$ (Fig. 1). Argininosuccinate was the most highly labeled species after 5 min and it was the first intermediate of Cit metabolism to decrease in ^{14}C-content over the time course. The ^{14}C in ArgS peaked at 45 min and in free Arg at 2 h, whereas ^{14}C continued to accumulate in protein up to 4 h. The ^{14}C in free Arg remained relatively constant from 2 to 4 h. At 4 h 97% of the ^{14}C-Cit was metabolized; 46% of this was recovered in protein Arg, 35% in free Arg, and 13% released as $^{14}CO_2$. Ninety-five to 100% of the ^{14}C in protein was accounted for in C-6 of Arg. No significant difference was found between the enzymic and HPLC determinations of the ^{14}C content of free Arg. Therefore, all the ^{14}C in free Arg was located in the C-6.

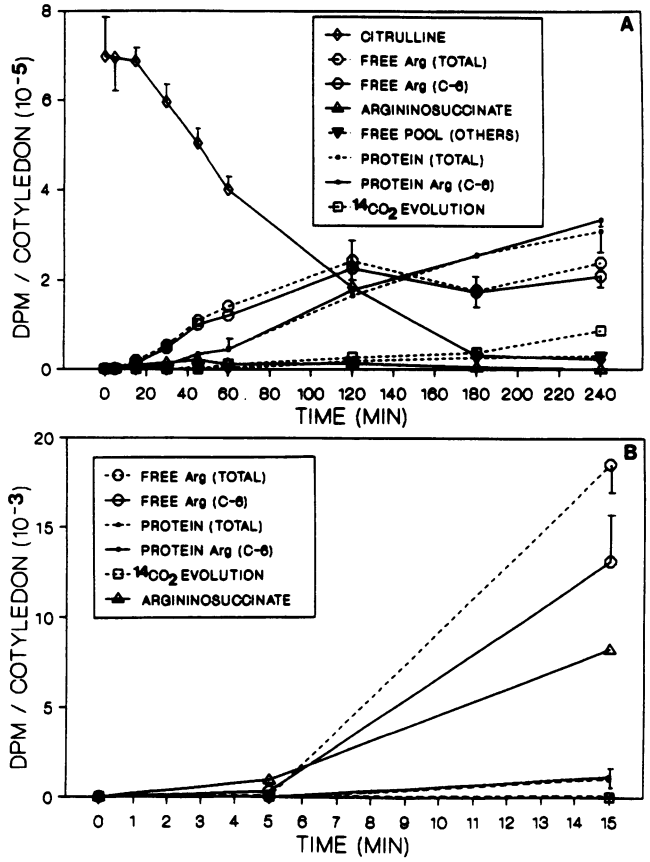

Figure 1. A, Distribution of ^{14}C with time among products of [ureido-^{14}C]Cit metabolism by an excised soybean cotyledon (23 DAA); B, distribution of ^{14}C among early products (excluding Cit) of [ureido-^{14}C]Cit metabolism (experiment is the same as Fig. 1A). The data were corrected for $^{14}CO_2$ evolution resulting from ^{14}C-urea impurities present in [ureido-^{14}C]Cit (1.2% of total ^{14}C). Total percent recovery for each time point was 88 to 100% of the ^{14}C injected at time zero. Values determined by HPLC are the mean of two injected cotyledons, whereas all other determinations represent the mean of three cotyledons. Vertical bars, ±1 SE and are not shown when smaller than the symbol.

Products of [1-^{14}C]Ornithine Metabolism

The metabolism of [1-^{14}C]Orn by the cotyledon resulted in ^{14}C incorporation into Cit, free and protein Arg, and in $^{14}CO_2$ evolution (Fig. 2). At 15 min Cit was the most highly labeled compound, and there was a lag in the incorporation of ^{14}C into free and protein Arg. In contrast to the ^{14}C-Cit feeding (Fig. 1), $^{14}CO_2$ was recovered after only 15 min (Fig. 2). The ^{14}C in Cit leveled off after 2 h, but the ^{14}C in free and protein Arg continued to accumulate up to 3 h. After 3 h, 15% of total ^{14}C was recovered as free Arg, 9% as protein Arg, 2% as Cit, and 1% was released as $^{14}CO_2$. Ninety-five to 100% of the ^{14}C in protein was in the C-1 of Arg, and there was no significant difference between the enzymic and HPLC determinations of ^{14}C in free Arg.

The addition of 200 mM unlabeled Cit to the ^{14}C-Orn feeding solution altered the labeling pattern (Fig. 3). After 5 min, Cit was the only labeled compound (2.6×10^4 dpm); this contrasts with the situation where unlabeled Cit was not

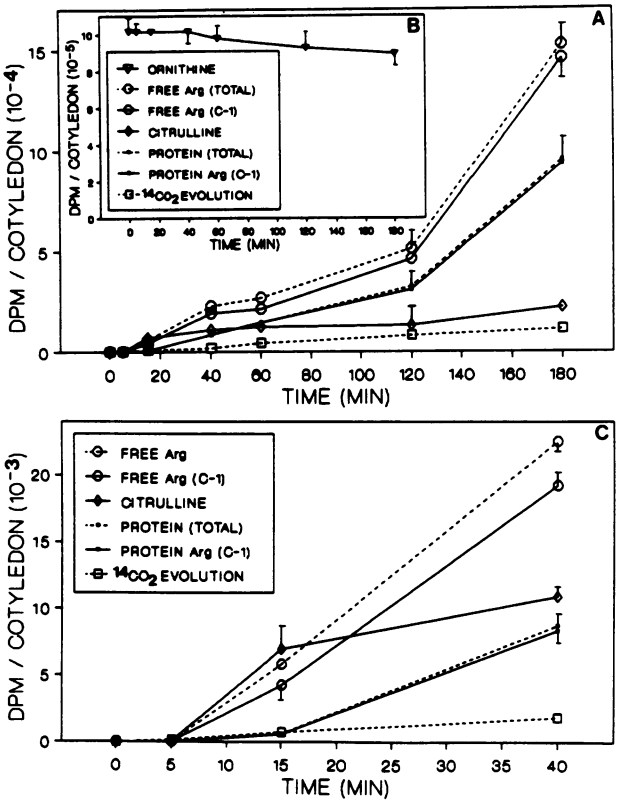

Figure 2. A, Distribution of ^{14}C with time among products of [1-^{14}C] Orn metabolism by an excised soybean cotyledon (23 DAA); B, depletion of ^{14}C-Orn with time; C, distribution of ^{14}C among early products (excluding Orn) of [1-^{14}C]Orn metabolism (experiment is the same as in Fig. 2A). Total percent recovery for each time point was 100% of the ^{14}C injected at time zero. Values determined by HPLC are the mean of two injected cotyledons, whereas all other determinations represent the mean of three cotyledons. Vertical bars, ±1 SE and are not presented when smaller than the symbol.

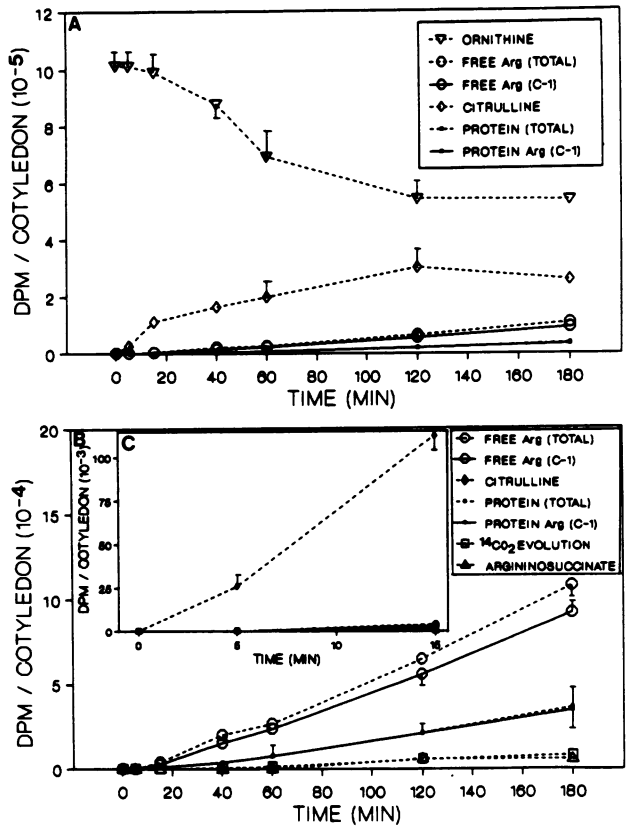

Figure 3. A, Distribution of ^{14}C with time among products of [1-^{14}C] Orn metabolism by an excised soybean cotyledon (23 DAA) in the presence of unlabeled Cit (800 nmol); B and C, the distribution of ^{14}C with time among early products (excluding ornithine) of [1-^{14}C]Orn metabolism (experiment is the same as in Fig. 3A). Total percent ^{14}C recovery for each time point was 93 to 100% of the ^{14}C injected at time zero. Values determined by HPLC are the mean of two injected cotyledons, whereas all other determinations represent the mean of three cotyledons. Vertical bars, ±1 SE and not shown when smaller than the symbol.

added (Fig. 2). The ^{14}C in Cit continued to increase, reaching a maximum at 2 h (3×10^5 dpm). Label was detected in ArgS at 2 and 3 h, in contrast to the control. The ArgS pool also increased from 11 nmol/cotyledon^{-1} to 22 nmol/cotyledon^{-1} over the 3 h incubation (data not shown). After 3 h 47% of the total ^{14}C-Orn was metabolized (Fig. 3), in contrast to the control where only 27% was utilized (Fig. 2). Twenty-six percent of the total ^{14}C was in Cit, 10% in free Arg, 3% in protein, 0.6% in ArgS, and 0.7% was evolved as $^{14}CO_2$ (Fig. 3). The addition of unlabeled Cit also decreased the ^{14}C content in protein by 63% and in Arg by 29%, and $^{14}CO_2$ evolution was decreased by 31%. Ninety-five to 100% of the ^{14}C in protein was in the C-1 of Arg, and no significant difference was found between the enzymic and HPLC determinations of ^{14}C in free Arg.

DISCUSSION

The aim of this study was to investigate the existence and nature of Arg biosynthesis in developing soybean cotyledons. Since it is believed that the urea cycle is involved (19), we have selected, based on our previous findings (13), cotyledons 23 DAA; at this stage of development the concentration of urea cycle intermediates is high and the cotyledons are easy to handle. In this investigation, the distribution of ^{14}C among products resulting from the metabolism of ^{14}C-labeled urea cycle intermediates was determined as a function of time; also, the recovery of applied ^{14}C-radioactivity was complete (Figs. 1–3). Thus, in contrast to other studies of Arg metabolism (1, 2, 4, 7–11, 17), we have provided convincing evidence from precursor-product relations for the reaction sequence leading to Arg.

Carbon-14 incorporation into protein from the metabolism of [ureido-^{14}C]Cit (Fig. 1) and [1-^{14}C]Orn (Fig. 2) exhibited an initial lag, followed by a steady increase over the remainder of the time course, indicating that protein was the end product in these experiments. This was preceded by the progressive accumulation of ^{14}C from Orn in free Arg (Fig. 2) or by the appearance and steady state labeling of free Arg with ^{14}C from Cit (Fig. 1). These results illustrate that in excised developing soybean cotyledons Arg is actively synthesized from urea cycle intermediates and that it is incorporated into cotyledonary protein. Similar findings have been reported previously for other plant tissues (1, 2, 4, 9, 11).

When ^{14}C-Cit was supplied, ArgS was the most highly labeled compound up to 5 min, and the first compound to lose ^{14}C with time (Fig. 1). Supplying ^{14}C-Orn resulted in the initial appearance of ^{14}C in Cit, which was also the first compound to reach a steady state ^{14}C level (Fig. 2). Addition of an excess of unlabeled Cit, together with ^{14}C-Orn, increased the ^{14}C recovery in Cit, delayed the appearance of ^{14}C in other products, including protein, and permitted the detection of ^{14}C in ArgS by doubling its pool size (Fig. 3; "Results"). An explanation for the apparent increase in the utilization of ^{14}C-Orn in the presence of exogenous Cit (*cf.* Figs. 2 and 3) is uncertain. Nevertheless, the precursor-product relations are consistent with the reaction sequence: ornithine → citrulline → argininosuccinate → arginine → protein arginine.

Injection of urea cycle intermediates also resulted in the evolution of $^{14}CO_2$; with ^{14}C-Cit, this occurred at a rate which was 28% of the rate of ^{14}C accumulation into protein Arg (Fig. 1). Since there is considerable urease (EC 3.5.1.5) activity in developing soybean seeds (16: BJ Micallef, BJ Shelp, unpublished data), it is possible that this $^{14}CO_2$ originated from ^{14}C-urea produced in the conversion of Arg to Orn by the enzyme arginase (EC 3.5.3.5). Unfortunately, arginase activity has not been studied in developing soybean seeds, but it has been found in developing pea seeds (6). In a companion paper (13) a balance sheet was developed, comparing the nitrogen composition of soybean seedcoat exudate and of cotyledonary protein and assuming that amino acids such as Arg, which were delivered in deficit supply in the vascular system, were directly incorporated into protein and not catabolized. The results in this study provide preliminary evidence that the catabolism of Arg, albeit at a lower rate than either biosynthesis or incorporation into protein, may also occur in developing soybean cotyledons.

In the present study ^{14}C-Orn appeared to be primarily used in Arg biosynthesis (Figs. 2 and 3). Carbon-14 was not found in Glu, and it could be assumed that ^{14}C was not incorporated into Pro because the ^{14}C recovery was always close to 100%. This is in contrast to previous work where label from Orn was detected in Arg, Pro, and Glu (2, 4). Therefore, the fate of the urea cycle intermediates, Orn and Cit, supports the idea that the major function of active biosynthesis of Arg in developing soybean cotyledons is to fulfill requirements for protein synthesis. However, it does not exclude the possibility that arginine or arginine-derived products are used to some extent in other metabolic pathways.

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