Plant Physiol. (1969) 44, 361–366

Metabolism of Arginine by Aging and 7 Day Old Pumpkin Seedlings

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Received October 21, 1968.

Abstract. The metabolism of arginine by etiolated pumpkin (*Cucurbita moschata*) seedlings was studied over various time and age intervals by injecting arginine-U-^{14}C into the cotyledons. At most, 25 % of the ^{14}C was transported from the cotyledon to the axis tissue and the amount of this transport decreased with increasing age of the seedlings. The cotyledons of 25 day old plants contained 60 % of the administered ^{14}C as unmetabolized arginine. Little ^{14}C was in sugars and it appeared that arginine was the primary translocation product. Time course studies showed that arginine was extensively metabolized and the labeling patterns suggest that different pathways were in operation in the axis and cotyledons. The amount of arginine incorporated into cotyledonary protein show that synthesis and turnover were occurring at rapid rate. Only 25 % of the label incorporated into protein by 1.5 hr remained after 96 hr. The label in protein was stable in the axis tissue. By 96 hr 50 % of the administered label occurred as $^{14}CO_2$ and it appeared that arginine was metabolized, through glutamate, by the citric acid cycle in the cotyledons. The experiments showed that an extensive conversion of arginine carbon into other amino acids did not occur.

During germination of a seed, an extensive breakdown of protein reserves occurs with a concomitant increase in amino acids. These amino acids provide nitrogen to synthesize new proteins and nucleic acids. Folkes and Yemm (8) have concluded that the major losses of glutamate, asparagine and proline in germinating barley could be accounted for in chlorophyll, other amino acids and other bases. However, although many amino acids were deficient in maize roots, these deficiences were not removed by other amino acids (14) and extensive conversions of the carbon from glutamate or leucine into those amino acids which were limiting did not occur (16). Rather the amino acid carbon was used in respiration or used directly in protein synthesis.

The reserve protein of pumpkin as in most dicotyledonous plants (4) and some trees (20) is characterized by a high arginine content (16 %) and this amino acid is released during germination (17). Boulter and Barber (5) have shown that arginine was rapidly transformed into proline and glutamate in *Vicia faba* and Kasting and Delwiche (10), using watermelon seedlings, showed that arginine was a precursor of ornithine cycle intermediates.

During germination, when pumpkin seedlings release considerable quantities of arginine, it may be expected that this amino acid would undergo extensive metabolic transformations if it were in excess of that required for protein synthesis. The fate of arginine-U-^{14}C injected into the cotyledons of germinating pumpkin seedlings is given in the present report.

Materials and Methods

Plant Material. Pumpkin seeds (*Cucurbita moschata* L. cultivar Dickinson field) were sown in moist vermiculite and maintained in a darkened germinator at 28° for various times. Seedlings were harvested by carefully removing the vermiculite from the root zone and used intact.

Incubation Procedure. L-Arginine-U-^{14}C was dissolved in distilled water to give a solution of 1 μmole having 7.5 μc of ^{14}C in 0.05 ml. This gave 10^6 cpm with our detection equipment. Paper chromatography revealed no other labeled compound in the sample.

L-Arginine-U-^{14}C was injected into each cotyledon (0.01 ml/plant) of 5 plants per duplicate sample. The samples were then placed in closed glass tubes covered with black polyethylene. Air was passed through a 50 % KOH scrubber and then through 0.1 M potassium phosphate, pH 6.7 in the glass tubes to aerate and prevent desication of the tissue. Respired $^{14}CO_2$ was carried in the air stream and bubbled through 10 ml of 20 % KOH in a 50 ml centrifuge tube. The absorbed CO_2 was converted to $BaCO_3$, filtered and the filter paper counted for radioactivity. The counts were corrected for background and self-absorption.

Analytical Methods. At predetermined times, the tissues were removed, rinsed with deionized water and transferred into 50 ml of boiling ethanol for 3 min. The ethanol was decanted and the tissues were ground with a Virtis Blender. The residues

were successfully extracted in boiling 80 % (v/v) ethanol, 40 % ethanol and again in 80 % ethanol. The extracts were combined and taken to dryness at 35° under reduced pressure.

The dried ethanol extract was extracted with ether (lipid fraction) and then dissolved in water or ethanol. This extract was then fractioned on Dowex resins into basic amino acids, acidic amino acids, neutral amino acids, organic acids, and sugars (18).

The ethanol insoluble fraction was hydrolized with 6 N HCl for 12 hr at 120° and then treated in the same manner as the ethanol extract.

The components of the fractions were separated by paper chromatography in *n*-butanol:propionic acid:water (623:310:437 v/v/v) (2), tert-butanol:methylethylketone:formic acid:water (40:30:15:15 v/v/v) (7) and *n*-butanol:90 % formic acid:water (1:1:1 v/v/v) aged 24 hr (3). After chromatography, the radioactive components were located by use of a strip counter.

Results

Following a 6 hr incubation with arginine-U-^{14}C, it was found that label was transported from the cotyledons to the axis tissue and that transport of this material from the cotyledon decreased as germination progressed in the dark (Fig. 1). Thus, during a 6-hr period, 17 % of the injected radioactive material was transported from the cotyledon on day 3 while seedlings germinated for 27 days transported only 7 % of the administered label to the axis tissue.

In the cotyledon tissue, lipid, water-soluble, and insoluble residues were labeled (table I). Maximal incorporation of labeled arginine into insoluble residue (protein) occurred in the cotyledon on the third day following germination. This period coincided with a period of rapid protein depletion from the cotyledons (22). Throughout the germination period there was an active incorporation of label into the insoluble residue of the cotyledon although the percentage of label in this fraction declined.

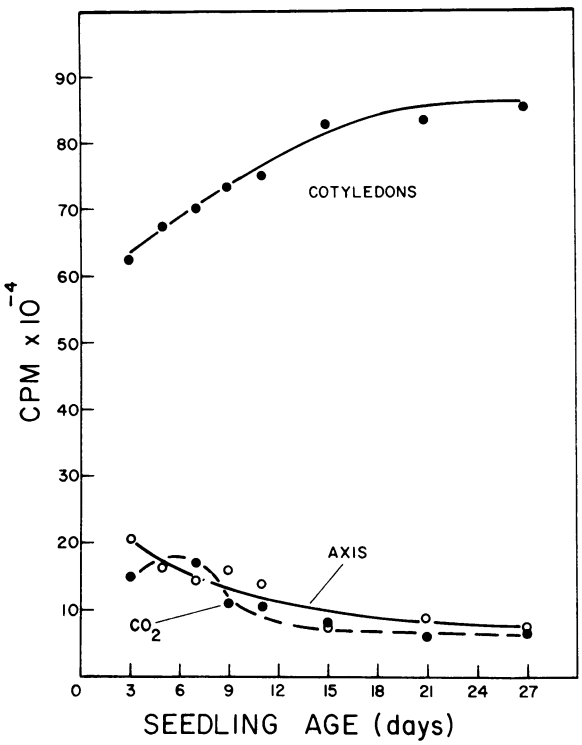

FIG. 1. Oxidation to $^{14}CO_2$ and translocation of arginine-^{14}C from the cotyledons to axis tissue of various aged pumpkin seedlings over a 6 hr period.

Analysis of the water-soluble material from the cotyledons of aging seedlings showed that a considerable redistribution of carbon had occurred. In young

Table I. *Distribution of ^{14}C from Arginine-U-^{14}C Metabolism by Cotyledons of Pumpkin*

Arginine-U-^{14}C (10^6 cpm) was injected into the cotyledons of pumpkin seedlings and incubated 6 hr.

Fraction	Seedling age (days)							
	3	5	7	9	11	15	20	25
				cpm × 10^{-3}				
Lipid	13.4	22.2	18.4	16.5	21.0	13.7	18.4	16.9
Sugars	7.3	9.4	9.1	11.1	6.8	2.4	2.2	1.5
Organic acids								
Citrate	2.5	1.0	1.3	2.5	3.8	4.6	3.8	2.1
Malate	4.3	7.4	8.0	16.3	18.7	28.2	20.0	26.7
α-Ketoglutarate	16.9	15.6	13.2	10.0	9.0	11.6	14.2	15.7
Succinate	0.5	0.8	0.9	2.0	2.5	4.2	3.4	3.6
Others	1.2	0.3	0.9	0.4	0	0	0	0
Amino acids								
Arginine	231.4	250.6	337.4	408.2	399.5	517.5	569.0	609.8
Ornithine	24.2	16.4	16.0	20.1	28.2	29.8	31.2	36.3
Glutamate	18.8	19.9	25.3	21.3	13.2	13.0	9.6	9.0
Aspartate	0	0.2	2.1	4.6	5.4	9.8	7.7	7.7
Neutrals	25.2	55.1	28.2	30.0	33.0	33.8	35.3	26.3
Insoluble residue	292.6	285.3	241.8	191.1	207.1	169.7	130.1	94.1

seedlings, the bulk of the ^{14}C in the organic acid fraction was found in α-ketoglutarate. The label in α-ketoglutarate then declined while the label in malate increased until 25 day old seedlings contained nearly twice as much ^{14}C in malate as α-ketoglutarate. Citrate and succinate were also labeled in the cotyledons.

In the amino acid fraction from the cotyledons (table I), the bulk of the radioactivity was found in arginine with some ^{14}C in ornithine, glutamate, aspartate and neutral amino acids. The transformation of arginine into ornithine and glutamate appeared to occur readily. The label remaining in arginine increased with germination time, and 25 day old cotyledons contained 60 % of the administered ^{14}C as unmetabolized arginine.

The transformations occurring in the cotyledon differed from those occurring in the axis. The bulk of the label in the organic acid fraction of the axis tissue was in malate at all germination times (table II), whereas in the cotyledons, as inverse relationship between malate and α-ketoglutarate existed. Labeled citrate was not detected in the axis tissue.

Although significant amounts of ^{14}C were found in the neutral amino acids and ornithine in the cotyledons, the axis tissue contained only traces of label in ornithine and there were indications that glutamate was converted to homoserine and γ-aminobutryic acid. The total amount of label in all the individual amino acids in the axis tissue declined with germination indicating that they were metabolized.

Label transported to the axis tissue was readily incorporated into the insoluble residue. In fact, after day 3, the label in the insoluble residue increased with germination until the eleventh and fifteenth day when there was a greater amount of label in the insoluble residue than the water soluble pool. The label in the residue then declined, suggesting that

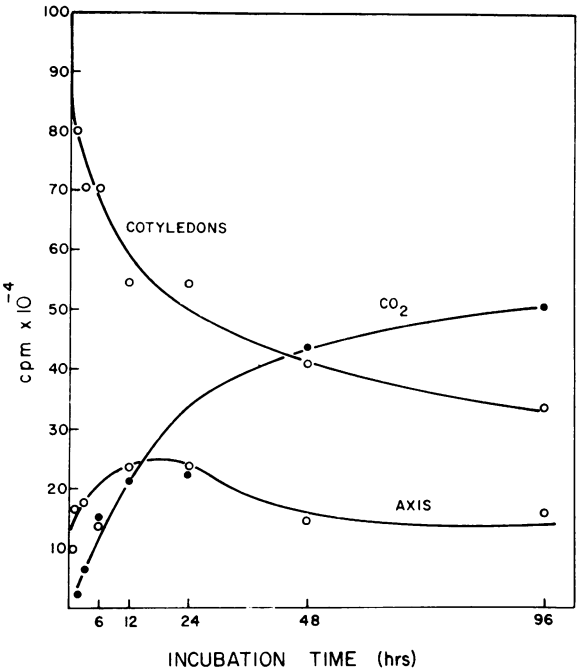

FIG. 2. Oxidation to CO_2 and translocation of arginine-^{14}C from the cotyledons to axis tissue, with time, of 7 day old pumpkin seedlings.

additional arginine-^{14}C was released and the increase in label in free arginine in the axis tissue at this time supports this suggestion. Little ^{14}C accumulated in sugars or lipid in the axis tissues.

Hydrolysis of the insoluble residues from both the axis and cotyledons of aging seedlings showed that the bulk of the ^{14}C was associated with arginine with minor activity occurring as glutamate. Traces of proline were found in the cotyledon insoluble residue. This is in agreement with the situation in

Table II. *Distribution of ^{14}C From Arginine-U-^{14}C Metabolism in Pumpkin Axis Tissue After Injection of Arginine-^{14}C Into the Cotyledons of Various Aged Plants*

Arginine-U-^{14}C (10^6 cpm) was injected into the cotyledons of pumpkin seedlings and incubated 6 hr.

Fraction	Seedling age (days)							
	3	5	7	9	11	15	20	25
	cpm × 10^{-3}							
Lipid	3.4	1.4	1.5	0.7	0.1	0.5	0.9	0.2
Sugars	3.2	7.1	3.3	1.7	1.2	0.6	0.3	0.2
Organic acids								
Malate	12.8	13.4	8.5	7.7	7.5	2.7	5.1	3.0
α-Ketoglutarate	4.8	4.6	2.9	2.8	2.0	0.8	1.7	1.7
Succinate	1.7	1.0	0.5	0.5	0.4	0.2	0.3	0.2
Amino acids								
Arginine	76.5	73.5	67.3	75.6	53.9	25.6	55.7	48.1
Glutamate	4.0	5.5	4.0	2.6	1.6	0.9	1.3	0.8
Aspartate	1.2	1.1	0.6	0.6	0.6	0.3	0.6	0.3
Others[1]	3.6	9.4	6.2	2.3	3.4	1.5	2.3	2.1
Insoluble residue	98.9	44.0	43.5	64.5	72.5	41.6	29.1	17.4

[1] Serine, glycine, homoserine, γ-aminobutyric acid, 2 unidentified nonprotein amino acids and traces of ornithine.

Table III. *The Metabolism of Arginine-^{14}C by Cotyledons of 7 Day Old Pumpkin Plants*

Arginine-U-^{14}C (10^6 cpm) was injected into the cotyledons of 7 day old pumpkin plants and incubated for the times shown.

Fraction	1.5 hr	3 hr	6 hr	12 hr	24 hr	48 hr	96 hr
				cpm × 10^{-3}			
Lipid	35.0	28.5	18.4	29.5	27.0	11.5	8.0
Sugars	9.1	9.9	9.1	8.4	5.6	6.2	3.8
Organic acids							
Citrate	3.9	3.5	1.3	2.4	1.1	1.3	1.3
Malate	20.1	10.5	8.5	4.3	3.0	2.9	1.8
α-Ketoglutarate	17.7	12.9	13.4	11.0	9.7	7.9	8.6
Succinate	4.6	1.9	1.5	0.3	0	0	0
Amino acids							
Arginine	359.9	339.7	359.5	276.6	308.5	278.6	219.7
Ornithine	57.2	40.0	18.0	0	0	0	0
Glutamate	14.5	12.1	19.4	7.0	5.5	2.7	5.4
Aspartate	1.3	1.0	1.8	0.8	1.0	0.4	0.5
Neutrals	11.3	10.0	9.9	7.2	7.1	6.0	7.4
Insoluble residue	275.0	222.5	241.8	194.5	188.0	100.0	77.5

the water-soluble material where the label in the protein amino acids was predominantly in arginine and glutamate.

With a 6 hr incubation, the transport of ^{14}C from the cotyledons to the axis tissue declined with increasing germination and longer incubation times with 7 day old seedlings were used to determine the ultimate fate of arginine. The pattern of redistribution of label from arginine-U-^{14}C injected into the cotyledons of 7 day old pumpkin seedlings is shown in Fig. 2. Increasing amounts of ^{14}C were removed from the cotyledons with time and after 96 hr only 35 % of the label remained in the cotyledons. This is in contrast to 70 % remaining after 6 hr. Increasing amounts of ^{14}C were released as CO_2 with 50 % of the label appearing as $^{14}CO_2$ by 96 hr. The amount of label in the axis tissues did not increase correspondingly however, and a maximum of 25 % of the radioactivity was found in this tissue. The percentage of label then declined to 15 % at 48 and 96 hr. This decline in ^{14}C indicates that some of the material transported from the cotyledons was released as CO_2 from the axis tissue.

Arginine was extensively metabolized in the cotyledons of 7 day old plants (table III). Approximately 50 % of the injected label was incorporated into the insoluble residue in the early phases of growth; however, the extent of this incorporation decreased as germination progressed. In fact, only 25 % of the label incorporated into the cotyledon residue by 1.5 hr remained after 96 hr, indicating that considerable turnover of this fraction was occurring. There was a continual decrease in radioactivity in the water-soluble components from the cotyledons with time and 50 % of the label was lost by 96 hr. Intermediates of the citric acid cycle were labeled and label in all of the organic acids declined with time. Arginine was rapidly but not extensively converted to other amino acids (table III) and the label in all of these amino acids declined with time, suggesting that they were either respired to CO_2 or transported to the axis tissue.

Table IV. *The Distribution of ^{14}C Within Pumpkin Axis Tissue After Injection of Arginine-U-^{14}C Into the Cotyledons of 7 Day Old Plants*

Arginine-U-^{14}C (10^6 cpm) was injected into the cotyledons of 7 day old pumpkin plants and incubated for the times shown.

Fraction	1.5 hr	3 hr	6 hr	12 hr	24 hr	48 hr	96 hr
				cpm × 10^{-3}			
Lipid	1.0	1.0	1.5	3.0	2.0	2.0	2.0
Sugars	1.8	1.9	3.3	5.7	6.9	4.7	4.2
Organic acids							
Citrate	0.5	0.5	0	0.6	0.4	0.2	0.2
Malate	4.9	9.0	8.5	18.7	11.3	6.7	5.7
α-Ketoglutarate	2.9	2.9	2.9	5.9	4.6	3.1	3.7
Succinate	0.9	1.1	0.5	0.5	0	0	0
Amino acids							
Arginine	92.6	74.4	67.5	110.5	124.3	75.4	86.1
Glutamate	9.2	15.3	4.0	10.7	7.7	4.3	3.8
Aspartate	1.4	3.5	0.6	1.4	1.0	0.5	0.4
Neutrals	8.8	10.4	6.0	6.5	6.8	4.1	4.4
Insoluble residue	43.5	49.5	45.5	72.0	67.5	45.5	50.0

In the cotyledons, α-ketoglutarate was an early recipient of ^{14}C and after 1.5 hr was the principally labeled organic acid (table III). In the axis tissue, however, malate was always the principally labeled organic acid (table IV). The label in malate and α-ketoglutarate increased up to 12 hr and then slowly declined. There was an increase in radioactivity in arginine up to 24 hr at which time the ^{14}C in this fraction declined. Arginine-^{14}C must have been metabolized to $^{14}CO_2$, as there was not a corresponding increase in the insoluble residue, other water-soluble components or lipid fractions in the axis tissue after 24 hr. The amount of radioactivity in the amino acids other than arginine, sugars and lipids followed a pattern similar to that found in arginine-^{14}C. Only traces of ornithine were found. The radioactivity in the insoluble residue of the axis tissue remained relatively stable over the experiment.

Hydrolysis of the insoluble residue from 7 day old seedlings showed that over 99 % of the ^{14}C was associated with arginine in both the cotyledon and the axis tissue.

Discussion

Previous workers have demonstrated that during germination there is an extensive mobilization of reserves from the cotyledons to the growing axis tissue. During germination of pumpkin seedlings considerable quantities of free arginine are found in the cotyledons (17) but the present results show that only 17 % of the ^{14}C from arginine-^{14}C was transported to the axis tissue in 6 hr (Fig. 1). This decrease in translocation with age suggests that the bulk of the reserve is mobilized soon after germination. This is in agreement with results with germinating peas in which 20 % of the ^{14}C from leucine-^{14}C was transported from the cotyledons to the axis tissue under similar conditions (1).

The labeling patterns between the cotyledon and axis tissue of aging seedlings (tables I and II) suggest that the bulk of the ^{14}C was transported as arginine. It appears that ornithine was not translocated from the cotyledons although it was present at all seedling ages. In castor bean, those protein amino acids which did not easily give rise to intermediates on the pathway of fat to sugar conversion were transported intact to the axis tissue (19).

The increase in arginine-^{14}C and malate-^{14}C in the cotyledons with age (table IV) and the decline in $^{14}CO_2$ (Fig. 1) suggests that as senescence approached more carbon was sequestered in pools not in ready equilibrium with degradative pathways. Lips and Beevers (12) have shown that corn roots contain at least 2 physically separated pools of malate. It is apparent that the metabolic activity of 25 day old seedlings was considerably less than that of 7 day old seedlings and Geronimo and Beevers (9) have shown that aging of pea leaves results in a decline in mitochondrial and glycolytic activity.

Larson and Beevers (11) have demonstrated the incorporation of labeled amino acids into pea cotyledonary protein during germination. The cotyledons of pumpkin incorporated a high percentage of radioactivity into protein at early seedling ages (table I) but with increasing age less ^{14}C occurred in protein and more remained as unmetabolized arginine-^{14}C. In the axis tissue (table II), however, the percentage of radioactivity in protein remained relatively stable although the total ^{14}C declined with age as less ^{14}C was transported to this tissue. This further emphasizes the difference in metabolic function between these 2 organs.

The protein reserve of pumpkin contains 16 % arginine (4) but the time course studies with 7 day old seedlings show that the bulk of the carbon from arginine was not transported to the axis tissue (Fig. 2). Folkes and Yemm (8) have concluded that the major losses of glutamate, asparagine and proline in germinating barley could be accounted for in chlorophyll, other amino acids and other bases. It is clear from Fig. 2, however that most of the ^{14}C from arginine-^{14}C is respired to $^{14}CO_2$ by pumpkin seedlings. Moreover, the decline in ^{14}C in the axis tissue after 24 hr, indicates that some of the ^{14}C transported to the axis tissue was released as $^{14}CO_2$. If additional radioactivity was transported to the axis tissue after 24 hr, this also must have been respired to $^{14}CO_2$. Although Wiley and Ashton (22) concluded that the bulk of the protein hydrolyzed in pumpkin cotyledons during the first 7 days of germination was transported to the axial tissue, it is apparent that the carbon and nitrogen of the arginine molecule do not always follow identical metabolic pathways. The nitrogen is transported and utilized in the axis tissues (22) but the carbon from arginine is metabolized principally to CO_2. The nitrogen derived from the catabolism of arginine is probably used in the synthesis of new amino acids and Sims and Folkes (15) have shown that the majority of amino acids are formed by transamination.

The labeling data from time course studies (tables III and IV) show that arginine was extensively metabolized in both the cotyledons and axis tissues. Stewart and Beevers (19) have shown that although the bulk of the carbon in castor beans is transported from the endosperm as sugar and the nitrogen is transported as glutamine, arginine was transported intact. The small amount of label in sugars indicate that the intermediates of the citric acid cycle arising from arginine metabolism are not in ready equilibrium with those of the glyoxylate cycle. It has been shown that these 2 pathways are located on 2 different organelles (6) and although the glyoxylate cycle occurs in pumpkin cotyledons, the labeling data show that arginine is closer metabolically to the mitochondria than the glyoxysomes.

Arginine was rapidly converted to ornithine, and arginase, the enzyme involved is known to be present in the cotyledons (17). Naylor (13) has shown that ornithine is easily converted to glutamate. Upon

deamination, glutamate yields α-ketoglutarate which readily enters the citric acid cycle (21). As neither arginosuccinic acid nor citrulline was detected, it is probable that arginine was respired to $^{14}CO_2$ by the suggested pathway.

Arginase was not found in the axis tissue (17) and only traces of ornithine were noted in the present study. However, the complete ornithine cycle is found in watermelon axis tissue (10) and it is probable that this cycle also exists in pumpkin axis tissue. However, the labeling pattern suggests that this pathway does not play a prominent role in arginine catabolism.

The cotyledons of 7 day old plants (table III) incorporated a high percentage of arginine into protein at a time when this fraction was being depleated (22). The decline in label in protein with time indicates that this fraction was turning over rapidly and that additional labeled arginine was released. However, the decline of ^{14}C in arginine with time, and no further increase in label in the axis tissue (Fig. 2) indicates that this additional arginine was rapidly metabolized to $^{14}CO_2$ in the cotyledons. In contrast with the cotyledons, the protein fraction in the axis tissue did not fluxuate greatly with time (table IV).

These studies show that although the reserve protein of pumpkin is high in arginine, an extensive conversion of arginine into other amino acids does not occur. Rather arginine is used directly in protein synthesis or the carbon is respired in the cotyledon. Small amounts are transported to the growing axis and the decline in label with time indicates that some of the arginine in the axis tissue is also catabolized to CO_2.

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