Plant Physiol. (1993) 103: 1195–1201

Regulation of Purine Metabolism in Intact Leaves of *Coffea arabica*[1]

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The capacity of *Coffea arabica* leaves (5- × 5-mm pieces) to synthesize de novo and catabolize purine nucleotides to provide precursors for caffeine (1,3,7-trimethylxanthine) was investigated. Consistent with de novo synthesis, glycine, bicarbonate, and formate were incorporated into the purine ring of inosine 5′-monophosphate (IMP) and adenine nucleotides (ΣAde); azaserine, a known inhibitor of purine de novo synthesis, inhibited incorporation. Activity of the de novo pathway in *C. arabica* per g fresh weight of leaf tissue during a 3-h incubation period was 8 ± 4 nmol of formate incorporated into IMP, 61 ± 7 nmol into ΣAde, and 150 nmol into caffeine (the latter during a 7-h incubation). Coffee leaves exhibited classical purine catabolism. Radiolabeled formate, inosine, adenosine, and adenine were incorporated into hypoxanthine and xanthine, which were catabolized to allantoin and urea. Urease activity was demonstrated. Per g fresh weight, coffee leaf squares incorporated 90 ± 22 nmol of xanthine into caffeine in 7 h but degraded 102 ± 1 nmol of xanthine to allantoin in 3 h. Feedback control of de novo purine biosynthesis was contrasted in *C. arabica* and *Cucurbita pepo*, a species that does not synthesize purine alkaloids. End-product inhibition was demonstrated to occur in both species but at different enzyme reactions.

The pathway for the de novo biosynthesis of purine nucleotides in vascular plants has been demonstrated to be similar to that previously described for bacteria and animals (Ross, 1981; Lovatt, 1983; Tomlinson and Lovatt, 1987). Carbon-2 and -8 originate in formate, the α carbon of Gly, or the β carbon of Ser. Carbon-6 is supplied by CO_2 and carbon-4 and -5 are from Gly (Ross, 1981; Lovatt, 1983). Anderson and Gibbs (1962) and Proiser and Serenkov (1963), respectively, demonstrated that the purine ring of caffeine (1,3,7-trimethylxanthine) was synthesized in a similar manner in leaves of coffee (*Coffea arabica*) and tea (*Camellia sinensis*). Despite this, to our knowledge, no study of either species has included examination of the activity or regulation of the pathway for the de novo biosynthesis of purine nucleotides in relation to the synthesis of caffeine.

In preparation for studies investigating caffeine biosynthesis, we assessed the capacity of leaves of *C. arabica* (cut into 5- × 5-mm squares) to synthesize purine nucleotides de novo by measuring the incorporation of radiolabeled precursors of the purine ring, provided at saturating concentrations to assess V_{max}, into IMP. In addition, we examined the further metabolism of IMP to adenine nucleotides and the degradation of the purine ring to allantoin and subsequently to urea. Feedback control of purine biosynthesis has not previously been demonstrated in plants (Ross, 1981). Because regulation of purine metabolism by end-product inhibition might be expected to be at a different enzymic step in a tissue producing purine alkaloids, we contrasted feedback control of purine metabolism in leaf squares of *C. arabica* with that of excised roots of summer squash (*Cucurbita pepo*), a species that does not synthesize purine alkaloids.

MATERIALS AND METHODS

Chemicals

All radiolabeled chemicals were purchased from ICN Pharmaceuticals, Inc., except [8-^{14}C]inosine, which was purchased from Amersham Corp. Liquiscint (liquid scintillation cocktail) was purchased from National Diagnostics. Mineral salts for both Shive's and Hoagland nutrient solutions were of analytical reagent quality and were purchased from Fisher Scientific Co. All other chemicals were purchased from Sigma Chemical Co.

Determination of Radioisotope Content

To determine the content of ^{14}C-radioisotope, samples were prepared as described in the text and then diluted with 6.5 mL of liquiscint for each 2 mL of sample. The content of radioisotope was measured using a Beckman LS 9000 liquid scintillation spectrometer. Each datum was corrected for quenching by an automatic external standardization system of quench analysis. A standard quench curve was determined by using a series of samples containing a known quantity of ^{14}C-radioactivity and an increasing amount of chemical quencher ($CHCl_3$). Data are presented as means ± SE with number of experiments given in parentheses.

[1] Supported in part by a fellowship from the Graduate Division, University of California, Riverside, and by the Citrus Research Center and Agricultural Experiment Station of the University of California, Riverside.

[2] This paper represents a portion of the dissertation submitted by G.M.N. in partial fulfillment of the requirements for a Ph.D. in Botany at the University of California.

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Abbreviations: dH_2O, distilled water; ΣAde, sum of the adenine nucleotides, adenosine, and adenine, obtained by converting the adenine nucleotides and adenosine to adenine by acid hydrolysis at 100°C; PRAICA, 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole.

Plant Material

Coffee (*Coffea arabica* L.) seedlings were donated by the Department of Agriculture in Puerto Rico and imported to California in accordance to U.S. Department of Agriculture regulations. The seedlings were transplanted in University of California soil mix and fertilized twice a week with half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950). Young, fully expanded leaves from trees that were 1 to 5 years old were used in all experiments. The leaves were collected just prior to the experiments, washed with dH_2O, and quickly blotted dry before weighing.

Seeds of summer squash (*Cucurbita pepo* L. cv Early Prolific Straightneck), courtesy of Harris-Moran Seeds, were allowed to imbibe in dH_2O at room temperature for 24 h. The seeds were rinsed three times with dH_2O, spread evenly on paper towels placed in a plastic box (33 × 23 × 9.5 cm), moistened with 7 mL of dH_2O, covered, and kept in the dark for 48 h at 30°C. After the seeds germinated, the roots (generally 2–4 cm long) were excised, pooled, and immediately weighed into 150-mg aliquots (approximately seven to eight roots) that were maintained between sheets of moistened paper towels until weighing was completed (Lovatt, 1983).

Incorporation of Radiolabeled Precursors into Acid-Soluble Purine Products

Conditions used for the incorporation of ^{14}C-labeled substrates of purine metabolism by plant tissues were essentially those described by Lovatt (1983). Leaf squares of *C. arabica* (500 mg fresh weight, midrib removed and cut into 5- × 5-mm pieces) or excised roots of *C. pepo* (150 mg fresh weight) were routinely preincubated for 2 h at 30°C in 5 mL of Shive's nutrient solution (Shive and Robbins, 1938) adjusted to pH 7.4. At the end of the preincubation period, the nutrient solution was decanted, and the leaf or root tissue was immediately transferred to fresh Shive's nutrient solution supplemented with one of the following radiolabeled precursors at the final concentration and specific radioactivity indicated: 25 mM [^{14}C]formate, 1800 dpm $nmol^{-1}$; 2 mM L-[1-^{14}C]Gly, 4000 dpm $nmol^{-1}$; 25 mM $NaH^{14}CO_3$, 2640 dpm $nmol^{-1}$; 2 mM DL-[3-^{14}C]Ser, 1730 dpm $nmol^{-1}$; 2 mM [6- or 8-^{14}C]inosine, 1100 dpm $nmol^{-1}$; 2 mM [6- or 8-^{14}C]adenosine, 1100 dpm $nmol^{-1}$; 2 mM [6- or 8-^{14}C]adenine, 1100 dpm $nmol^{-1}$; 2 mM [6- or 8-^{14}C]hypoxanthine, 200 dpm $nmol^{-1}$; 2 mM [6- or 8-^{14}C]xanthine, 400 dpm $nmol^{-1}$; 2 mM [^{14}C]urea, 765 dpm $nmol^{-1}$; and any other additives in a final volume of 5 mL, pH 7.4.

Reaction mixtures were incubated for 3 or 7 h at 30°C in a water bath shaker. Incubations were carried out in 25-mL Erlenmeyer flasks, each sealed with a rubber stopper fitted with a plastic center well containing a filter paper wick. Just before the reactions were terminated, 0.3 mL of 20% (w/v) KOH was injected into the center well. When catabolism was being measured, 20% (w/v) KOH was added to the center wells at the beginning of the incubation period. At the end of the incubation, the center wells were transferred quantitatively to scintillation vials containing 3.7 mL of water, and the content of radiolabel was determined as described above. The reactions were terminated by injecting 1 mL of 6 N $HClO_4$ or 15 mL of 95% (v/v) ethanol into the main chamber of the flask for the subsequent isolation of purine nucleotide products or caffeine, respectively.

The contents of the main chamber of the flask were homogenized with a Polytron homogenizer (PCU-2, speed 6; Brinkman Instruments), and the insoluble material was removed by centrifugation at 10,000*g* for 10 min at 0°C.

Isolation of Radiolabeled Purine Nucleotide Products by Co-Crystallization

IMP and adenine nucleotides and adenosine synthesized by leaf or root tissue from radiolabeled precursors were converted by acid hydrolysis to hypoxanthine and adenine, respectively, by heating the acid-soluble fraction at 100°C for 1 h prior to neutralization with KOH. Hypoxanthine, adenine, xanthine, and allantoin were isolated from the neutralized acid-soluble fraction by co-crystallization with carrier (Lovatt, 1983; Tomlinson and Lovatt, 1987).

Isolation of Radiolabeled Caffeine by Co-Crystallization and Purification by TLC

Reactions terminated with 15 mL of 95% (v/v) ethanol were made 80% ethanolic and saturated at the boiling point with 3 g of commercial caffeine. The saturated solutions were allowed to cool slowly to room temperature and then to 4°C overnight in a refrigerator. The crystals were collected by suction filtration and washed with 80% (v/v) ethanol. The crystals were dissolved and purified further by TLC on Eastman Kodak 13254 cellulose sheets with fluorescent indicator 6065, using *n*-propanol:ammonia:H_2O (100:60:40, v/v/v) as the developing system. The commercial standard was located with a UV light, and the corresponding area from the chromatogram of the sample was cut out and eluted with 4 mL of 0.25 N KOH for determination of the content of radioisotope.

Isolation of Radiolabeled PRAICA by TLC

PRAICA synthesized by leaf or root tissue was isolated from the neutralized acid-soluble fraction by TLC on Eastman Kodak 13254 cellulose with fluorescent indicator 6065 using two different solvent systems described by Ciardi and Anderson (1968). Solvent system A consisted of 95% (v/v) ethanol:1 M ammonium acetate (pH 7.9) (75:30, v/v). Solvent system B was composed of isobutyric acid:0.5 N ammonia (pH 3.6) (10:6, v/v). The commercial standard of PRAICA was located with a UV light, and the corresponding area from the chromatogram of the sample was cut out and eluted with 4 mL of 0.5 N HCl for determination of the content of radioisotope.

Recovery of Radiolabeled Carbon-8 of Adenine, Hypoxanthine, and Xanthine in [^{14}C]Urea

The amount of [^{14}C]urea synthesized from [8-^{14}C]purine bases by coffee leaves was quantified according to the method of Lovatt and Cheng (1984). A 2-mL aliquot was removed from the acid-soluble supernatant solution and adjusted to pH 7.5 with KOH and made 50 mM with 17 mg of $Ba(OH)_2$.

The resulting precipitate was removed by centrifugation in a tabletop centrifuge (Dynac) at setting 70 for 10 min. The supernatant was made 100 mM Gly with 1 M Gly-NaOH buffer, pH 9.0, and brought to a final pH of 9.0 with NaOH. The final volume of the sample was recorded. A 1-mL aliquot was transferred to a 25-mL Erlenmeyer flask to which was added 0.2 mL of an aqueous solution containing two units of urease (EC 3.5.1.5) (Sigma type III, from jackbeans). All flasks were sealed with a rubber stopper fitted with a plastic center well containing a filter paper wick and 0.3 mL of 20% (w/v) KOH and incubated in a water bath shaker for 3 h at 37°C. The reaction was terminated by injecting $HClO_4$ into the main chamber of the flask to a final concentration of 1 N. Flasks were returned to the water bath shaker for 30 min to allow the $^{14}CO_2$ released by urease to distill from the acidified incubation mixture into the KOH in the center well. A 0.2-mL aliquot of 0.1 N $NaHCO_3$ was injected into the main chamber of the flask to flush any residual $^{14}CO_2$ from the incubation mixture. After an additional 30 min at 37°C in the water bath shaker, the center well and its contents were transferred to a scintillation vial for determination of the content of radioisotope.

RESULTS

Demonstration of de Novo Biosynthesis of Purine Nucleotides in Leaves of *C. arabica*

Evidence that leaves of *C. arabica* synthesize purine nucleotides via the classical pathway found in other eukaryotic and prokaryotic organisms (Lehninger, 1981) was provided by demonstration of the incorporation of the radiolabeled carbons of formate, bicarbonate, and Gly into ΣAde. Formate and bicarbonate, but not Gly, were supplied at a saturating concentration, i.e. the one resulting in V_{max}. The rate of incorporation of all three precursors was similar: 47 ± 7 nmol ($n = 3$) g^{-1} fresh weight of leaf tissue during a 3-h incubation period. Adenine nucleotides and adenosine synthesized during the incubations were subjected to acid hydrolysis and isolated as adenine, confirming that the labeled carbons were incorporated into the adenine ring. Ser is an effective donor of carbon for the synthesis of N^5,N^{10}-methenyltetrahydrofolate by well-known reversal of the reaction catalyzed by Ser hydroxymethyltransferase (Lovatt, 1983; Mitchell et al., 1986). The capacity of coffee leaves to utilize the β carbon of Ser in the de novo biosynthesis of purine nucleotides was confirmed by demonstration of the incorporation of 27 nmol of [3-^{14}C]Ser into ΣAde g^{-1} fresh weight of leaf tissue during a 3-h incubation period.

Azaserine, a known inhibitor of de novo purine biosynthesis in plants (Lovatt, 1983; Tomlinson and Lovatt, 1987), inhibited by about 60% the incorporation of [^{14}C]bicarbonate into ΣAde in *C. arabica* leaves when provided at concentrations from 5 to 20 mM.

In addition, 8 ± 4, 4 ± 0, and 25 ± 5 nmol ($n = 2$) of [^{14}C]formate, [^{14}C]bicarbonate, and [^{14}C]Gly, respectively, were incorporated into IMP, the first true purine nucleotide, per g fresh weight of leaf tissue during a 3-h incubation period. IMP was subjected to acid hydrolysis and isolated as hypoxanthine, confirming that the radiolabeled carbons were incorporated into the purine ring.

Demonstration of the Pathway for Purine Catabolism in Intact Cells of Leaves of *C. arabica*

Evidence for the operation of the classical pathway for purine catabolism in leaves of *C. arabica* is provided by the following observations. Both [^{14}C]formate and [^{14}C]adenosine were incorporated into hypoxanthine at a rate of 10 and 1306 nmol g^{-1} fresh weight of leaf tissue during a 7-h incubation period, respectively.

The radiolabeled ribosides, inosine and adenosine, and bases, adenine and hypoxanthine, were converted into xanthine (Table I). This conversion was partially inhibited by the addition of 10 mM allopurinol (1H-pyrazolo[3,4-*d*]pyrimidine-4-ol), a known inhibitor of xanthine oxidase (xanthine:oxygen oxidoreductase; EC 1.1.3.22) and xanthine dehydrogenase (xanthine:NAD oxidoreductase; EC 1.2.1.37) (Fujihara and Yamaguchi, 1978; Triplett et al., 1980), which catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid (Table I). In this and subsequent experiments, significance should not be attached to the variations in sensitivity of precursor incorporation to the presence of the inhibitor or added intermediate. Such variations probably arise from differences in the rate of uptake and metabolism of the individual precursors. Consistent with hypoxanthine being an intermediate in the conversion of inosine, adenosine, and adenine to xanthine, addition of 10 mM hypoxanthine to the preincubation and incubation mixtures reduced the specific radioactivity of ^{14}C-labeled inosine, adenosine, and adenine incorporated into xanthine (Table I).

Radiolabeled allantoin was isolated from *C. arabica* leaf squares incubated with [8-^{14}C]hypoxanthine and [8-^{14}C]xanthine. The rate of incorporation of radiolabeled precursor into allantoin was reduced by added xanthine or uric acid (Table II). Both steps catalyzed by either xanthine oxidase or xanthine dehydrogenase were inhibited by allopurinol (Table II).

Despite a high rate of xanthine degradation (102 ± 1 [$n = 2$] nmol converted to allantoin g^{-1} fresh weight of leaf tissue during a 3-h incubation), coffee leaf squares incorporated 90 ± 22 ($n = 3$) nmol of xanthine into caffeine g^{-1} fresh weight of leaf tissue during a 7-h incubation period.

By the classical pathway of purine degradation, CO_2 is generated from carbon at position 6 of the purine ring when uric acid is converted to allantoin. Radiolabeled CO_2 was

Table I. *The effect of hypoxanthine and allopurinol on the incorporation of radiolabeled inosine, adenosine, adenine, and hypoxanthine into xanthine by leaves of C. arabica*

8-^{14}C-Labeled Precursor (2 mM)	Incorporation of Precursor into Xanthine		
	Control	+Allopurinol (10 mM)	+Hypoxanthine (10 mM)
	nmol g^{-1} fresh wt (7 h)$^{-1}$	*% control*	
Inosine	341	57	21
Adenosine	3030	75	78
Adenine	3264	15	50
Hypoxanthine	3890	44	

Table II. *Effect of xanthine, uric acid, and allopurinol on the incorporation of radiolabeled hypoxanthine and xanthine into allantoin in leaves of C. arabica*

8-^{14}C-Precursor (2 mM)	Incorporation of Precursor into Allantoin[a]		
	Additions (10 mM)	Experiment 1	Experiment 2
		% control	
Hypoxanthine	Xanthine	3	5
	Uric acid	41	36
	Allopurinol	7	7
Xanthine	Uric acid	58	29
	Allopurinol	8	6

[a] Incorporation of [8-^{14}C]hypoxanthine and [8-^{14}C]xanthine into allantoin in the controls (no additions = 100%) was 4,423 ± 23 and 40,955 ± 363 (n = 2) dpm g^{-1} fresh weight during a 3-h incubation period, respectively.

recovered from coffee leaf squares incubated with [6-^{14}C]-inosine, [6-^{14}C]adenosine, [6-^{14}C]adenine, [6-^{14}C]hypoxanthine, or [6-^{14}C]xanthine. Xanthine (10 mM final concentration) added to the preincubation and incubation periods reduced the amount of $^{14}CO_2$ released (Table III). In all cases, 10 mM allopurinol inhibited the generation of $^{14}CO_2$ (Table III). Furthermore, 7, 113, and 316 nmol of [^{14}C]urea were formed from [8-^{14}C]adenine, [8-^{14}C]hypoxanthine, and [8-^{14}]-xanthine, respectively, g^{-1} fresh weight of leaf tissue during a 3-h incubation. The amount of [^{14}C]urea isolated underestimates the amount produced during the 3-h incubation period because of the presence of urease in leaves of *C. arabica*. Urease activity was demonstrated by the release of 120 ± 9 nmol (n = 2) of $^{14}CO_2$ from exogenously supplied [^{14}C]urea g^{-1} fresh weight of leaf tissue during a 3-h incubation.

The possibility that the incorporation observed reflected the activity of fungal or bacterial contamination of the coffee leaves is considered unlikely because no growth of microorganisms was detected after incubation of leaf squares on potato dextrose agar in either the dark or light for 60 h at 30°C. Further evidence that the metabolic activities measured were those of intact cells of coffee leaves and not those of fungal or bacterial contaminants was obtained by comparing the release of $^{14}CO_2$ from [8-^{14}C]hypoxanthine by leaf squares with that generated by an equal amount of leaf squares homogenized with the Polytron tissue homogenizer at speed 6. Intact leaf squares degraded 62 nmol of hypoxanthine to CO_2 g^{-1} fresh weight of leaf tissue; virtually no $^{14}CO_2$ was generated by the homogenate.

End-Product Inhibition of the Purine de Novo Pathway by Added Adenosine or Its Metabolites

The effect of added adenosine on the activity of the de novo purine pathway of *C. arabica* was contrasted with *C. pepo*, a species that does not synthesize purine alkaloids. Using the same assay conditions used with *C. arabica*, we confirmed that excised squash roots do not synthesize caffeine. One gram fresh weight of coffee leaves incorporated 32 nmol of [^{14}C]bicarbonate into caffeine during a 3-h incubation period, whereas no radioactivity above background was isolated as caffeine from incubations with squash roots. By using various radiolabeled precursors of IMP, we determined which enzymes of the de novo purine pathway were sensitive to end-product inhibition.

Adenosine (3 mM final concentration) or its metabolites inhibited by at least 90% the incorporation of [^{14}C]bicarbonate or [^{14}C]formate into IMP and ΣAde and by 30 to 40% into hypoxanthine in excised roots of *C. pepo* (Table IV). In the presence of added adenosine, [^{14}C]bicarbonate and [1-^{14}C]Gly, but not [^{14}C]formate, accumulated in PRAICA (Table V). Failure of [^{14}C]formate to accumulate in PRAICA in the presence of adenosine is consistent with the β carbon of Ser being the biological source of carbon-8 of the purine ring (Lehninger, 1981). In the absence of added adenosine, incorporation of formate into PRAICA was 2- to 10-fold lower than incorporation of bicarbonate and Gly in both species (see legend to Table V). Taken together, the results with *C. pepo* show that end-product inhibition occurs at one or both of the enzymic reactions converting PRAICA to IMP.

For coffee leaves, 10 mM adenosine or its metabolites inhibited the incorporation of [^{14}C]bicarbonate into PRAICA by about 30% as well as into IMP, ΣAde, and hypoxanthine by 30 to 60% (Tables IV and V). Consistent with end-product inhibition at an early step in the pathway leading to PRAICA formation, exogenous adenosine inhibited by 30% the incorporation of [1-^{14}C]Gly into PRAICA (Table V). As observed with excised roots of *C. pepo*, added adenosine had little or no effect on the incorporation of [^{14}C]formate into PRAICA (Table V), but unlike squash, it resulted in the accumulation of [^{14}C]formate in IMP, ΣAde, and hypoxanthine (Table IV). Accumulation of [^{14}C]formate in IMP, ΣAde, and hypoxanthine indicates that there is no regulation in coffee leaves at either of the enzymic steps converting PRAICA to IMP. However, added adenosine inhibited by 50% the incorporation of [^{14}C]formate into caffeine. The interpretation that end-product regulation might also occur at xanthine oxidase or xanthine dehydrogenase is equivocal. The increase in the pool size of IMP and adenine nucleotides and/or adenine or hypoxanthine that would result from exogenous adenosine would also result in the observed accumulation of label from [^{14}C]formate and reduction in the specific radioactivity of [^{14}C]formate incorporated into caffeine.

Table III. *Effect of xanthine and allopurinol on the catabolism of purine ribonucleosides and bases in leaves of C. arabica*

6-^{14}C-Precursor (2 mM)	nmol of $^{14}CO_2$ Generated g^{-1} Fresh Wt of Leaf Tissue during a 7-h Incubation period		
	Control	+Xanthine (10 mM)	+Allopurinol (10 mM)
Inosine	9 ± 0.1[a]	5 ± 0.3	6 ± 0.2
Adenosine	6 ± 0.2	3 ± 0.4	3 ± 0.1
Adenine	40 ± 0.3	18 ± 0.0	30 ± 0.7
Hypoxanthine	29 ± 2.1	17 ± 0.1	22 ± 1.0
Xanthine	35 ± 1.0		6 ± 0.2

[a] Mean ± SE, n = 2.

Table IV. *Effect of adenosine on the incorporation of radiolabeled bicarbonate and formate into IMP, ΣAde, and hypoxanthine by leaf squares of C. arabica and excised roots of C. pepo*

^{14}C-Precursor	Product	Incorporation of Precursor into IMP, ΣAde, and Hypoxanthine[a]			
		Squash		Coffee	
		Control	+Adenosine (3 mM)	Control	+Adenosine (10 mM)
		dpm g⁻¹ fresh wt (3 h)⁻¹	*% control*	*dpm g⁻¹ fresh wt (3 h)⁻¹*	*% control*
Bicarbonate	IMP	7,346	4	8,384	43
	ΣAde	644,160	12	88,032	51
	Hypoxanthine	12,911	69	8,803	73
Formate	IMP	17,126	3	13,081	247
	ΣAde	28,098	8	122,207	144
	Hypoxanthine	5,422	58	7,613	359

[a] Separate experiments were conducted to assess the incorporation of precursors into each product. Radiolabeled biocarbonate (25 mM, 2640 dpm $nmol^{-1}$) and formate (5 mM, 2050 dpm $nmol^{-1}$) were typically provided at the final concentration and specific radioactivity given in parentheses. Each datum is the average from two separate experiments. The differences between any two replicates did not exceed 10% of the mean.

DISCUSSION

The occurrence of the complete purine pathway for de novo biosynthesis of adenine nucleotides was established in leaves of *C. arabica* by demonstrating that the ^{14}C-precursors known to donate carbons for the synthesis of the purine ring (Gly, Ser, formate, and bicarbonate) radiolabeled IMP and ΣAde. IMP and the adenine nucleotides were isolated as their respective bases, confirming that the radiolabeled carbons were incorporated into the purine ring. Sensitivity of the pathway to azaserine, a known inhibitor of de novo purine biosynthesis in microorganisms, animals, and plants, was demonstrated.

Radiolabeled bicarbonate and formate were incorporated into IMP at the rate of 4 ± 0 and 8 ± 4 (n = 2) nmol, respectively, and into ΣAde at the rate of 42 ± 2 and 61 ± 7 (n = 2) nmol, respectively, g^{-1} fresh weight of leaf tissue during a 3-h incubation period. The lower level of incorporation of both [^{14}C]bicarbonate and [^{14}C]formate into IMP compared to ΣAde is consistent with the occurrence of IMP as an intermediate in the de novo synthesis having a faster turnover rate and/or a smaller pool size than ΣAde. This was also the case for young fruit of the Washington navel orange (*Citrus sinensis*). Incorporation of [^{14}C]bicarbonate into IMP was 50% less than into ΣAde (Tomlinson and Lovatt, 1987).

Evidence demonstrating purine degradation via the classical pathway has been previously reported for plants (Reinbothe and Mothes, 1962; Kalberer, 1965; Baumann and Wanner, 1972; Baumann, 1975; Larsen and Jochimsen, 1987). Operation of this pathway in leaves of *C. arabica* was demonstrated by incorporation of the radiolabeled purine ribosides, inosine and adenosine, and bases, adenine and hypoxanthine, into xanthine and the recovery of radiolabeled carbon-6 of these compounds as $^{14}CO_2$. Radiolabeled carbon-8 of hypoxanthine and xanthine was isolated in allantoin. In addition, allopurinol, a known inhibitor of xanthine oxidase and xanthine dehydrogenase, in all cases inhibited the incor-

Table V. *Effect of adenosine on the incorporation of radiolabeled Gly, bicarbonate, and formate into PRAICA by leaf squares of C. arabica and excised roots of C. pepo*

^{14}C-Precursor	Incorporation of Precursor into PRAICA[a] in the Presence of Adenosine			
	Squash		Coffee	
	A	B	A	B
	% control			
Gly	128	130	72	73
Bicarbonate	155	180	69	72
Formate	99	100	111	117

[a] In the absence of adenosine (control; = 100%), incorporation of radiolabeled Gly, bicarbonate, and formate into PRAICA isolated by solvent system A (A) was, respectively, 260, 417, and 102 nmol g^{-1} fresh weight during a 3-h incubation for squash roots and 370, 196, and 67 nmol g^{-1} fresh weight during a 3-h incubation for coffee leaf squares. Incorporation of radiolabeled Gly, bicarbonate, and formate into PRAICA isolated by solvent system B (B) was, respectively, 216, 1237, and 57 nmol g^{-1} fresh weight during a 3-h incubation for excised squash roots and 677, 120, and 79 nmol g^{-1} fresh weight during a 3-h incubation for coffee leaf squares. Adenosine was supplied at 3 or 10 mM final concentration for experiments using excised squash roots or coffee leaf squares, respectively. Each datum is the average for two separate experiments for each solvent system used. The differences between any two replicates did not exceed 10% of the mean.

poration of radiolabeled precursors into allantoin. Radiolabeled carbon-8 of adenine, hypoxanthine, and xanthine was recovered in [^{14}C]urea. Taken together, the results provide conclusive evidence that purine degradation in coffee leaves proceeds via the classical pathway.

Our results are consistent with a previous report (Waller et al., 1983) that coffee converts xanthine to urea, but they are in contrast to those of Winkler et al. (1985, 1987, 1988), who provided evidence that urea is not produced by the major pathway of allantoin catabolism in soybean (*Glycine max* L. Merr.). In our hands, coffee leaf squares degraded 316 nmol of [8-^{14}C]xanthine to urea g^{-1} fresh weight in 3 h. Because of significant urease activity in leaves of *C. arabica*, the amount of urea resulting from purine degradation is an underestimate.

Leaf squares of *C. arabica* incorporated 42 nmol of [^{14}C]-bicarbonate into ΣAde g^{-1} fresh weight during a 3-h incubation. In contrast, the capacity of young fruit of the Washington navel orange to incorporate [^{14}C]bicarbonate into ΣAde assessed by the same method was 20 nmol g^{-1} fresh weight in 5 h, whereas roots excised from 2-d-old *C. pepo* seedlings incorporated 244 nmol of [^{14}C]bicarbonate g^{-1} fresh weight in 3 h. Evidence has been accumulating since 1980 in support of the role of the classical de novo purine pathway in ureide biosynthesis in nodules of *Vigna unguiculata* L. Walp. and *G. max* L. Merr. Slices of cowpea nodules incorporated [^{14}C]Gly into allantoin to a greater degree than labeled Glc or acetate (Atkins et al., 1980). $^{14}CO_2$ was incorporated into the carbon-6 position of xanthine by soybean nodules and accumulated in the presence of added allopurinol (Boland and Schubert, 1982). Boland et al. (1982) demonstrated that enzymes of purine biosynthesis were associated with the proplastid fraction of soybean nodules. Subsequently, Boland and Schubert (1983) established that this fraction incorporated radiolabeled Gly, Ser, and bicarbonate into IMP, AMP, inosine, and XMP. Concurrently, Shelp and Atkins (1983) demonstrated that cell-free extracts of nitrogen-fixing cowpea nodules incorporated [^{14}C]Gly into formylglycinamide ribonucleotide, aminoimidazole carboxamide ribonucleotide, IMP, inosine, hypoxanthine, XMP, xanthosine, and xanthine. Cell-free extracts of cowpea nodules displayed activity 100-fold higher than we report for *C. arabica* (Atkins et al., 1982). In contrast, incorporation rates for nodules from amide-exporting legumes (Atkins et al., 1982) and for cotyledons and embryonic axes from 1- and 2-d-old *Phaseolus mungo* seedlings (Nobusawa and Ashihara, 1983) were similar to those we report here.

The incorporation of radiolabeled bicarbonate into IMP and ΣAde was inhibited by the addition of adenosine to excised roots of *C. pepo* and leaf squares of *C. arabica*, suggesting that the purine de novo pathway was regulated by end-product inhibition in both species. In an attempt to confirm feedback control of the pathway and identify the site of inhibition in *C. pepo* and *C. arabica*, the influence of added adenosine on the incorporation of carbon donated by the various precursors of the purine ring into PRAICA, IMP, ΣAde, and hypoxanthine was quantified. The results of these experiments revealed that exogenous formate was not an effective donor of carbon-8 of the purine ring in either plant species. Thus, added adenosine had little or no effect on the incorporation of [^{14}C]formate into PRAICA in coffee or squash. Although in vitro studies have not yet been conducted in plants to determine the nature of the transformylases and their one-carbon folate substrates, our results are consistent with the β carbon of Ser being the biological source of carbon-8 of the purine ring (Lehninger, 1981).

For excised roots of *C. pepo*, adenosine or one of its metabolites inhibited by at least 90% the incorporation of [^{14}C]-bicarbonate or [^{14}C]formate into IMP and ΣAde and by 30 to 40% into hypoxanthine with concomitant accumulation of [^{14}C]bicarbonate and [1-^{14}C]Gly, but not [^{14}C]formate, in PRAICA. These results provide evidence that regulation of purine de novo biosynthesis by feedback inhibition occurs at one or both of the enzymic reactions converting PRAICA to IMP.

In contrast, for leaf squares of *C. arabica*, adenosine or one of its metabolites inhibited the incorporation of [^{14}C]bicarbonate or [1-^{14}C]Gly, but not [^{14}C]formate, into PRAICA, suggesting that regulation of purine de novo biosynthesis by feedback inhibition occurs at a step prior to the synthesis of PRAICA. We were unable to determine the specific site of regulation. It is interesting to note that glutamine-5-phosphoribosylamine:PPi phosphoribosyltransferase (amido-phosphoribosyltransferase; EC 2.4.2.14), which catalyzes the first reaction of de novo purine biosynthesis, the formation of phosphoribosylamine from 5-phosphoribosyl-1-PPi and Gln, has been demonstrated when highly purified to be sensitive to end-product inhibition by adenine and guanine nucleotides for bacteria, yeast, and mammals (Messenger and Zalkin, 1979) and by IMP, XMP, and GMP in soybean root nodules (Reynolds et al., 1984). Results obtained for *C. arabica* are consistent with feedback inhibition of amidophosphoribosyltransferase. Although end-product inhibition at any of the seven remaining enzymic reactions leading to the synthesis of PRAICA would also produce the observed results, no other step sensitive to feedback regulation between phosphoribosylamine and IMP, including Ser hydroxymethyltransferase, has been demonstrated in the prokaryotic, avian, mammalian, or plant species examined (Wyngaarden, 1976; Lehninger, 1981; Ross, 1981; Mitchell et al., 1986). Our results are the first to demonstrate end-product regulation of the de novo purine pathway in intact plant cells and the first to demonstrate regulation at one or both of the enzymic reactions converting PRAICA to IMP in any species.

ACKNOWLEDGMENTS

The authors thank Anne Cheng for her capable technical assistance and Drs. Monica Madore and James Sims for their valuable comments during the development of this research.

Received February 19, 1993; accepted August 5, 1993.
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