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Separate de Novo and Salvage Purine Pools Are Involved in the Biosynthesis of Theobromine but Not Caffeine in Leaves of *Coffea arabica* L.[1]

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In *Coffea arabica* leaves, the purine ring of theobromine (3,7-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) is provided by de novo purine biosynthesis: (a) [^{14}C]glycine, [^{14}C]bicarbonate, and [^{14}C]formate were incorporated into inosine 5′-monophosphate (IMP), sum of adenine nucleotides (ΣAde), theobromine, and caffeine; and (b) incorporation of [^{14}C]formate into IMP, ΣAde, theobromine, and caffeine was inhibited by azaserine, a known inhibitor of de novo purine biosynthesis. Capacity of coffee leaves to salvage added purines was demonstrated by incorporation of [^{14}C]hypoxanthine into ΣAde and the incorporation of [^{14}C]adenosine, [^{14}C]adenine, [^{14}C]inosine, and [^{14}C]hypoxanthine into both theobromine and caffeine. Consistent with synthesis of theobromine from two separate purine nucleotide pools, one synthesized de novo and one via salvage, added xanthine 5′-monophosphate (XMP), inosine, or hypoxanthine failed to reduce the incorporation of [^{14}C]formate into theobromine but diluted the specific radioactivity of [^{14}C]adenosine and [^{14}C]adenine incorporated into theobromine. Evidence that theobromine is not the immediate precursor of caffeine is provided: (a) [^{14}C]xanthine was incorporated into caffeine but not into theobromine; (b) exogenous xanthine diluted the specific radioactivity of caffeine synthesized from [^{14}C]adenine and [^{14}C]hypoxanthine but caused accumulation of radiolabel in theobromine; (c) allopurinol, a known inhibitor of the conversion of hypoxanthine to xanthine, reduced incorporation of [^{14}C]adenine and [^{14}C]hypoxanthine into caffeine but caused accumulation of radiolabel in theobromine; and (d) incorporation of [^{14}C]formate into caffeine, but not into theobromine, was reduced by added XMP, inosine, or hypoxanthine.

Attempts to elucidate the pathway by which theobromine (3,7-dimethylxanthine) is synthesized in *Coffea arabica* have been limited exclusively to studies of the metabolism of preformed nucleosides and bases or their 1-, 3-, or 7-methylated counterparts (Looser et al., 1974; Suzuki and Takahashi, 1975a; Roberts and Waller, 1979; Suzuki and Waller, 1984). The results of these studies demonstrated that radiolabeled adenine, guanine, and xanthine were incorporated into 7-methylxanthine, theobromine, and caffeine (Suzuki and Waller, 1984; Negishi et al., 1985c), whereas the radiolabeled ribosides were first incorporated into 7-methylxanthosine and then into 7-methylxanthanine, theobromine, and caffeine (Negishi et al., 1992). Both 7-methylxanthosine and 7-methylxanthine were methylated to form theobromine and caffeine, but 7-methylinosine and 7-methylguanosine were not (Looser et al., 1974; Baumann and Wanner, 1977). In view of these results, there is much speculation regarding the substrate of the first methylation step and subsequent enzyme reactions leading to the synthesis of theobromine (Ogutuga and Northcote, 1970a, 1970b; Baumann and Wanner, 1977; Baumann et al., 1978; Roberts and Waller, 1979; Waller et al., 1983; Suzuki and Waller, 1984; Suzuki et al., 1992). In addition, the relationship between theobromine and caffeine (1,3,7-trimethylxanthine) remains equivocal. Paraxanthine (1,7-dimethylxanthine) was a better substrate for methylation than theobromine or theophylline in the synthesis of caffeine in both *C. arabica* (Roberts and Waller, 1979; Ashihara and Nobusawa, 1981) and *Camellia sinensis* (Suzuki and Takahashi, 1975a). However, both coffee fruit and tea leaves incorporated 1- and 7-[$^{14}CH_3$]xanthine predominantly into theophylline and theobromine, respectively; incorporation into paraxanthine was negligible (Suzuki and Takahashi, 1975a; Roberts and Waller, 1979).

Additional evidence that theobromine is an immediate precursor of caffeine was provided by studies using tea leaves: (a) radiolabeled adenine (Ashihara and Kubota, 1986; Fujimori and Ashihara, 1990; Fujimori et al., 1991), guanine, and hypoxanthine (Suzuki and Takahashi, 1976a, 1976b; Ashihara and Kubota, 1987) were incorporated into theobromine and caffeine to a greater extent than into theophylline by intact leaves; (b) during leaf development, theobromine content decreased as the concentration of caffeine increased (Ashihara and Kubota, 1986); and (c) cell-free extracts of tea leaves converted 7-methylxanthosine and 7-methylxanthine to both theobromine and caffeine (Suzuki and Takahashi, 1975a; Negishi et al., 1985a, 1985b; Fujimori et al., 1991).

In the present study, we contrasted the role of purines produced endogenously via the de novo pathway in the synthesis of theobromine and caffeine with that of purines

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Abbreviations: 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; XMP, xanthine 5′-monophosphate.

generated via the various salvage or catabolic reactions from exogenously supplied pools. We also addressed the specific question of whether theobromine is the immediate precursor of caffeine in coffee leaves. To undertake this study, we developed two new methods for isolating and quantifying the amount of theobromine and caffeine synthesized from radiolabeled precursors by intact cells of leaves of *C. arabica*. Each method, with the proof of its reliability, is reported in this communication.

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.

Determination of Radioisotope Content

To determine the content of 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 the means ± SE with the number of experiments given in parentheses.

Plant Material

Coffee (*Coffea arabica* L.) seedlings were donated by the Department of Agriculture in Puerto Rico and imported to California in accordance with 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 distilled H_2O, and quickly blotted dry before weighing.

Incorporation of Radiolabeled Precursors into Purine Nucleotides, Theobromine, and Caffeine

Leaves of *C. arabica* (500 mg fresh weight, midrib removed and cut in 5- × 5-mm pieces) were routinely preincubated for 2 h at 30°C in 5 mL of Shive's nutrient solution (Shive and Robbins, 1938) [5 mM $Ca(NO_3)$, 2 mM $MgSO_4$, 2 mM K_2SO_4, 1 mM KH_2PO_4, 1 mg of Fe L^{-1}, 0.13 mg of Cl L^{-1}, 1 mg of Mn L^{-1}, 0.1 mg of Zn L^{-1}, 0.1 mg of B L^{-1}, 0.01 mg of Cu L^{-1}, and 0.01 mg of Na L^{-1}] adjusted to pH 7.4. At the end of the preincubation period, the nutrient solution was decanted, and the leaf 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: 5 mM [^{14}C]formate, 3300 dpm $nmol^{-1}$; 2 mM [1-^{14}C]Gly, 400 dpm $nmol^{-1}$; 25 mM [^{14}C]-bicarbonate, 2640 dpm $nmol^{-1}$; 2 mM [8-^{14}C]inosine, 1100 dpm $nmol^{-1}$; 2 mM [8-^{14}C]adenosine, 1100 dpm $nmol^{-1}$; 2 mM [8-^{14}C]adenine, 1100 dpm $nmol^{-1}$; 2 mM [8-^{14}C]theophylline, 150 dpm $nmol^{-1}$; 2 mM [1-$^{14}CH_3$]caffeine, 200 dpm $nmol^{-1}$; [8-^{14}C]hypoxanthine, 200 dpm $nmol^{-1}$; 2 mM [8-^{14}C]-xanthine, 400 dpm $nmol^{-1}$; 2 mM L-[$^{14}CH_3$]Met, 1540 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 to absorb any $^{14}CO_2$ released during the incubation. The reactions were then 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 theobromine 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 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 and adenine were isolated from the neutralized acid-soluble fraction by co-crystallization with carrier (Lovatt, 1983; Tomlinson and Lovatt, 1987).

Isolation of Radiolabeled Theobromine and Caffeine by Co-Crystallization

The supernatant fraction from the reactions terminated with $HClO_4$ was neutralized with KOH, and the resulting precipitate of $KClO_4$ was removed by centrifugation at 10,000*g* for 10 min at 0°C. The neutralized acid-soluble supernatant fraction was brought to 125 mL with 0.01 N HCl and saturated at the boiling point with 1 g of commercial theobromine. The supernatant fraction from the reactions terminated with 85% (v/v) ethanol was made 80% (v/v) ethanolic and saturated at the boiling point with 3 g of commercial caffeine. The saturated solutions of theobromine and caffeine 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 recrystallized to a constant specific radioactivity per 20 mg of crystals dried to a constant weight at 60°C.

The reliability of the methods for isolating radiolabeled theobromine and caffeine synthesized by coffee leaves from [^{14}C]formate or radiolabeled purine ribosides and bases was

Table I. *Incorporation of radiolabeled Gly, bicarbonate, and formate into IMP, ΣAde, theobromine, and caffeine in leaves of C. arabica*

Conditions for the incorporation of 100 mM [^{14}C]Gly, 400 dpm nmol^{-1} were optimal; no attempts were made to determine optimal conditions for 25 mM [^{14}C]bicarbonate, 2640 dpm nmol^{-1} or 5 mM [^{14}C]formate, 3300 dpm nmol^{-1}.

^{14}C-Precursor	Incorporation of Precursor into:			
	IMP	ΣAde	Theobromine	Caffeine
	nmol g^{-1} fresh wt (3 h)$^{-1}$		nmol g^{-1} fresh wt (7 h)$^{-1}$	
Gly	25 ± 5[a]	37 ± 5	14 ± 1	135 ± 10
Bicarbonate	4 ± 0	42 ± 2	7 ± 1	32 ± 2
Formate	8 ± 4	61 ± 7	31 ± 7	147 ± 13

[a] Mean ± SE, $n = 2$.

assessed using two different methods. The purity of theobromine and caffeine crystals recrystallized to a constant specific radioactivity as described above was determined by comparing the specific radioactivity of the crystals before and after TLC on Eastman Kodak 13254 cellulose with fluorescent indicator 6065 using *n*-propanol:ammonia:H_2O (100:60:40, v/v/v) as the developing solvent (Ashihara and Nobusawa, 1981). The commercial standards of theobromine and caffeine were located with a UV light, and, in each case, the corresponding area of the chromatogram of the sample was cut out and diluted with 4 mL of 0.25 N KOH for determination of radioisotope content.

In addition, potential contamination by radiolabeled purine ribosides or bases was assessed by attempting to isolate the various ^{14}C-precursors, provided at the concentrations and specific radioactivities used in the incorporation studies, from a mock neutralized acid-soluble fraction or ethanol-soluble fraction without plant tissue by co-crystallization with theobromine and caffeine, respectively.

RESULTS

Demonstration of the de Novo Biosynthesis of the Purine Ring of Theobromine and Caffeine in Intact Cells of *C. arabica*

Evidence that leaves of *C. arabica* synthesize purine nucleotides and purine alkaloids de novo was provided by demonstration of the incorporation of the radiolabeled carbons of Gly, bicarbonate, and formate into IMP, ΣAde, theobromine, and caffeine (Table I).

IMP and ΣAde were subjected to acid hydrolysis, and the resulting hypoxanthine and adenine, respectively, were shown to be radioactive, thus confirming that the radiolabeled carbons were incorporated into the purine ring. In addition, azaserine, a known inhibitor of de novo purine biosynthesis that is effective in plants (Lovatt, 1983; Tomlinson and Lovatt, 1987), inhibited the incorporation of [^{14}C]-formate into ΣAde by 40% and into IMP, theobromine, and caffeine by 30%.

The reliability of the methods for the isolation of labeled theobromine and caffeine by co-crystallization with commercial carrier was verified by two separate techniques. The radiolabeled metabolite of [^{14}C]formate isolated by co-crystallization with carrier theobromine or caffeine was shown to co-chromatograph with its respective commercial standard. In addition, chromatography of either carrier containing the metabolite of [^{14}C]formate did not result in a significant change in its specific radioactivity. The specific radioactivity of theobromine and caffeine before chromatography was 620 and 910 dpm per 20 mg of dry weight of crystals, respectively, and 555 and 692 dpm per 20 mg of crystals, respectively, after chromatography. Radioactivity above background level was not found in any other regions of the chromatograms. Recoveries of approximately 80% are typical; no adjustment in the data was made to approximate 100% recovery.

Potential contamination of isolated theobromine and caffeine by radiolabeled purine ribosides and bases was assessed by isolating these radiolabeled purine precursors from a mock neutralized acid-soluble fraction or ethanol-soluble fraction without leaf tissue by co-crystallization with commercial carrier. With the exception of a low level of contamination (20%) of theobromine by [^{14}C]caffeine, none of the radiolabeled precursors used in this study co-crystallized with carrier theobromine or caffeine (Table II).

[^{14}C]Caffeine was not used as a substrate in any assays, and although the specific radioactivity of caffeine synthesized by leaf tissue would be significantly lower than that used to test for contamination, care was taken in interpreting all experiments assessing incorporation of ^{14}C-precursors into theobromine.

Optimal Conditions for Measuring the Incorporation of Radiolabeled Precursors into Theobromine and Caffeine in Intact Cells

Assays routinely used 500 mg fresh weight of leaf tissue cut into 5- × 5-mm squares (midrib removed) suspended in 5 mL of Shive's nutrient solution, pH 7.4. The optimal concentration of [^{14}C]Gly incorporated into IMP and into

Table II. *Absence of contamination of theobromine and caffeine by radiolabeled purine ribosides and bases*

Precursors were supplied as: 100 mM [^{14}C]Gly, 400 dpm nmol^{-1}; 2 mM [^{14}C]adenine, 1100 dpm nmol^{-1}; 2 mM [^{14}C]xanthine, 400 dpm nmol^{-1}; 2 mM [^{14}C]caffeine, 200 dpm nmol^{-1}; or 2 mM [^{14}C]-theophylline, 150 dpm nmol^{-1}.

^{14}C-Precursor	Percentage Recovery[a] of Radiolabeled Precursors Isolated by Co-Crystallization with Commercial Carrier	
	Theobromine	Caffeine
Gly	0.4	0.5
Adenine	0.9	0.6
Xanthine	0.4	0.0
Caffeine	19.4	
Theophylline	1.1	2.5

[a] Total dpm isolated by co-crystallization with commercial theobromine and caffeine divided by the initial dpm supplied in the mock neutralized acid-soluble and ethanol-soluble fractions, respectively, without plant tissue times 100%.

caffeine was 100 mM. This concentration was used to determine the effect of the duration of the incubation period on caffeine biosynthesis. Maximum incorporation of [^{14}C]Gly into caffeine was 345,660 dpm g^{-1} fresh weight of leaf tissue obtained with a 7-h incubation period at 30°C. Preincubation of the leaf tissue for 2 or 3 h at 30°C in Shive's nutrient solution doubled the incorporation of [^{14}C]Gly into caffeine during the subsequent 7-h incubation period. Thus, all studies reported here in which incorporation into purine alkaloids was assessed used a 2-h preincubation period, followed by an incubation period of 7 h in fresh Shive's nutrient solution supplemented with a radiolabeled precursor. Under these assay conditions, the rate of caffeine biosynthesis obtained with 5 mM [^{14}C]formate was equal to that obtained with 100 mM [^{14}C]Gly, and the rate of theobromine biosynthesis was doubled over that obtained with the saturating concentration of Gly (Table I). Thus, formate was used in all assays in which the role of the de novo pathway in the synthesis of purine alkaloids was examined. Assays measuring the incorporation of radiolabeled precursors into IMP or ΣAde used a 2-h preincubation period followed by a 3-h incubation period previously shown to be optimal (Lovatt, 1983).

The possibility that the incorporation observed reflected the activity of fungal or bacterial contamination of the coffee leaves is considered unlikely since 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.

Evidence that Separate de Novo and Salvage Purine Pools Are Involved in the Biosynthesis of Theobromine in Leaves of *C. arabica*

The incorporation of [^{14}C]formate into theobromine was significantly reduced by the addition of adenosine or adenine to the preincubation and incubation mixtures but not by the addition of the ribonucleotide XMP, the riboside inosine, or the bases hypoxanthine and xanthine (Table III). In contrast, XMP, inosine, adenine, and hypoxanthine added to the preincubation and incubation mixtures at a final concentration of 10 mM reduced the specific radioactivity of [^{14}C]adenosine and [^{14}C]adenine incorporated into theobromine (Table IV). The ability of coffee leaves to utilize inosine and hypoxanthine for the synthesis of theobromine was confirmed by demonstrating that 39 nmol of [^{14}C]inosine and 50 nmol of [^{14}C]hypoxanthine were incorporated into theobromine g^{-1} fresh weight of tissue during a 7-h incubation period (Table V). There was no significant incorporation of [^{14}C]xanthine into theobromine—approximately 1 nmol was incorporated g^{-1} fresh weight of leaf tissue during a 7-h incubation period (Table V). In addition, coffee leaves exhibited the capacity to convert 236 nmol of [^{14}C]hypoxanthine into ΣAde g^{-1} fresh weight during a 3-h incubation period.

Failure of XMP, inosine, and hypoxanthine to dilute the specific radioactivity of [^{14}C]formate incorporated into theobromine while diluting the specific radioactivity of [^{14}C]adenosine and [^{14}C]adenine incorporated into theobromine is consistent with the synthesis of theobromine from two separate purine pools: one generated endogenously via the de novo biosynthetic pathway and the other by the salvage of exogenously supplied purines (Fig. 1). The results provide evidence that exogenously supplied adenosine and adenine are available to both pools, whereas adenine, adenosine, or adenine nucleotides synthesized from other exogenously supplied purines do not enter the de novo pool.

Evidence that Theobromine and Caffeine Are the Products of Separate Pathways in Leaves of *C. arabica*

In contrast to the lack of effect by added purines on the incorporation of [^{14}C]formate into theobromine, with the exception of adenosine and adenine, incorporation of [^{14}C]-formate into caffeine was reduced in the presence of added XMP, inosine, adenosine, adenine, hypoxanthine, and xanthine (Table III).

In addition, coffee leaves metabolized xanthine in a different manner in relation to theobromine and caffeine. Incorporation of [^{14}C]xanthine into theobromine was negligible (Table V). Added unlabeled xanthine (10 mM final concentration) increased incorporation of radiolabeled inosine, adenine, adenosine, and hypoxanthine into theobromine (Table V). Allopurinol, a known inhibitor of degradation of hypoxanthine and xanthine, supplied at final concentration of 10 mM, either increased or had no effect on the incorporation of these precursors into theobromine (Table V). In contrast, 90 ± 22 (n = 3) nmol of [^{14}C]xanthine were incorporated into caffeine g^{-1} fresh weight of coffee leaves during a 7-h incubation period. Consistent with xanthine being an intermedi-

Table III. *The effect of XMP, inosine, adenosine, adenine, hypoxanthine, and xanthine on the incorporation of radiolabeled formate into theobromine and caffeine in leaves of C. arabica*

No attempts were made to determine optimal conditions for the incorporation of 5 mM [^{14}C]formate, 3300 dpm nmol^{-1}.

^{14}C-Precursor	Additions (10 mM)	Incorporation of Precursor into Product[a]	
		Theobromine	Caffeine
		% *control*	
Formate	XMP	113	45
	Inosine	135	90
	Adenosine	48	47
	Adenine	19	50
	Hypoxanthine	110	49
	Xanthine	110	73

[a] Results are the means of two to three separate experiments. Values for each experiment did not vary from the mean by more than 10%. Incorporation of [^{14}C]formate into theobromine and caffeine averaged 102,300 and 485,100 dpm g^{-1} fresh weight, respectively, during a 7-h incubation period.

Table IV. *The effect of XMP, inosine, adenosine, adenine, and hypoxanthine on the incorporation of radiolabeled adenosine and adenine into theobromine and caffeine in leaves of C. arabica*

No attempts were made to determine optimal conditions for the incorporation of 2 mM [8-^{14}C]adenosine, 1100 dpm nmol^{-1}, or 2 mM [8-^{14}C]adenine, 1100 dpm nmol^{-1}.

^{14}C-Precursor	Additions (10 mM)	Incorporation of Precursor into Product[a]	
		Theobromine	Caffeine
		% control	
Adenosine	XMP	50	62
	Inosine	40	90
	Adenine	4	74
	Hypoxanthine	63	35
Adenine	XMP	82	68
	Inosine	87	90
	Adenosine	28	10
	Hypoxanthine	74	53

[a] Results are the means of one to two separate experiments. Values for each experiment did not vary by more than 20%. An average of 132,000 dpm of [^{14}C]adenosine and 113,300 dpm of [^{14}C]adenine were incorporated into theobromine and 381,700 dpm of [^{14}C]adenosine and 188,100 dpm of [^{14}C]adenine were incorporated into caffeine per g fresh weight during a 7-h incubation period.

ate in the synthesis of caffeine, unlabeled xanthine (10 mM final concentration) reduced the specific radioactivity of caffeine formed from radiolabeled formate, adenosine, adenine, and hypoxanthine (Table VI). Allopurinol (10 mM final concentration) inhibited the incorporation of radiolabeled formate, adenine, and hypoxanthine into caffeine by more than 50% (Table VI).

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

No attempts were made to determine optimal conditions for the incorporation of 2 mM [8-^{14}C]inosine, 1100 dpm nmol^{-1}; 2 mM [8-^{14}C]adenosine, 1100 dpm nmol^{-1}; 2 mM [8-^{14}C]adenine, 1100 dpm nmol^{-1}; 2 mM [8-^{14}C]hypoxanthine, 200 dpm nmol^{-1}; or 2 mM [8-^{14}C]xanthine, 400 dpm nmol^{-1}.

^{14}C-Precursor	Incorporation of Precursor into Theobromine			
	Control		+Xanthine (10 mM)	+Allopurinol (10 mM)
	nmol g^{-1} (7 h)$^{-1}$[a]		% control[b]	
Inosine	39	(1)	151	98
Adenosine	84 ± 34	(2)	130	121
Adenine	103	(1)	116	95
Hypoxanthine	50 ± 5	(3)	138	126
Xanthine	1 ± 0.8	(3)		

[a] Mean ± SE (n = 2–3); number of experiments is given in parentheses. [b] Values are the averages, as indicated for the control, of two to three separate experiments. The values obtained did not vary from the mean by more than 20%.

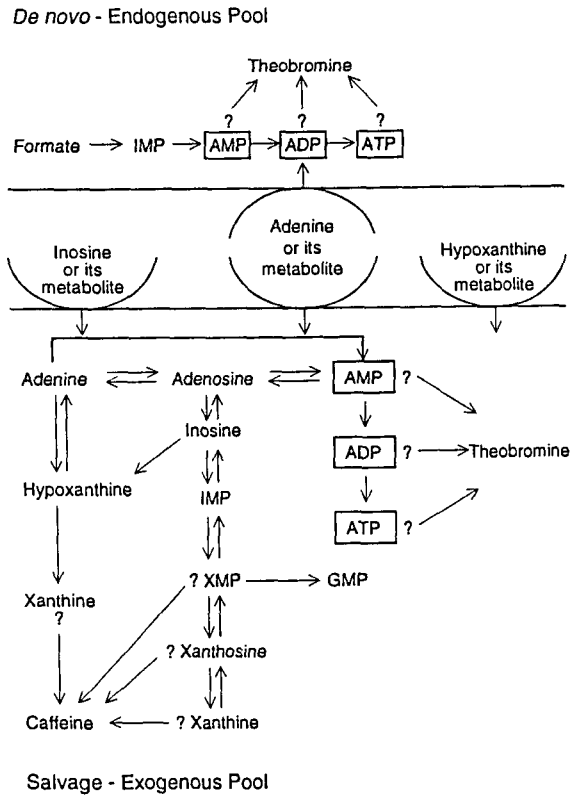

Figure 1. Proposed compartmentalization of two separate pools of purines for the biosynthesis of theobromine in leaves of *C. arabica*, one generated endogenously by the de novo pathway and the other from salvage of exogenously supplied purines, ribosides, and bases.

The effect of various purines on the incorporation of L-[^{14}CH$_3$]Met into theobromine and caffeine was assessed. Incorporation into theobromine was 130 and 160% of the control value when 3-methyladenine and 7-methylxanthosine were provided in the reaction mixture. In contrast, the incorporation of L-[^{14}CH$_3$]Met into caffeine was significantly enhanced by two different monomethylpurines, 1-methylguanosine (141% of control) and 1-methylinosine (130% of control). However, when supplied exogenously, all three dimethylxanthines increased the incorporation of L-[^{14}CH$_3$]-Met into caffeine: paraxanthine (223% of control) > theobromine (216%) > theophylline (130%).

Taken together, the results of this research provide strong evidence that endogenously synthesized theobromine is not the immediate precursor of caffeine in actively metabolizing leaves of *C. arabica*.

DISCUSSION

Leaves of *C. arabica* were demonstrated to have the capacity to synthesize the purine ring of theobromine and caffeine de novo, utilizing radiolabeled formate, bicarbonate, or Gly or by salvage of exogenously supplied adenosine, adenine, inosine, or hypoxanthine. The capacity of coffee leaves to synthesize theobromine or caffeine from [^{14}C]adenine was

Table VI. *The effect of xanthine and allopurinol on the incorporation of radiolabeled formate, inosine, adenosine, adenine, and hypoxanthine into caffeine in leaves of C. arabica*

No attempts were made to determine optimal conditions for the incorporation of 5 mM [8-^{14}C]formate, 3300 dpm nmol^{-1}; 2 mM [8-^{14}C]inosine, 1100 dpm nmol^{-1}; 2 mM [8-^{14}C]adenosine, 1100 dpm nmol^{-1}; 2 mM [8-^{14}C]adenine, 1100 dpm nmol^{-1}; or 2 mM [8-^{14}C]hypoxanthine, 200 dpm nmol^{-1}.

^{14}C-Precursor	Incorporation of Precursor into Caffeine			
	Control		+Xanthine (10 mM)	+Allopurinol (10 mM)
	nmol g^{-1} fresh wt (7 h)$^{-1}$[a]		% control[b]	
Formate	147 ± 13	(2)	73	56
Inosine	49	(1)	226	89
Adenosine	347 ± 46	(2)	56	124
Adenine	171	(1)	65	45
Hypoxanthine	157 ± 30	(2)	26	37

[a] Mean ± SE (n = 2); number of experiments is given in parentheses. [b] Values are the averages (where appropriate) for two separate experiments. The values obtained did not vary from the mean by more than 20%.

significantly greater than reported previously for coffee fruit: compare 103 and 171 nmol of [^{14}C]adenine incorporated into theobromine and caffeine, respectively, g^{-1} fresh weight of leaf tissue during a 7-h incubation period in this study to 0.3 and 2.0 nmol of [^{14}C]adenine incorporated into theobromine and caffeine, respectively, g^{-1} of immature coffee fruit during a 36-h incubation period (Suzuki and Waller, 1984). The low rate of incorporation observed for coffee fruit may be due to the duration of the incubation period. For leaves, maximum incorporation of radiolabeled precursor into caffeine was obtained with a 7-h incubation period. There was a significant decrease in the amount of caffeine isolated after 24 h of incubation and a 90% reduction after 48 h.

Although Anderson and Gibbs (1962) and Proiser and Serenkov (1963) previously showed that the purine ring of caffeine was synthesized de novo in *C. arabica* and *C. sinensis*, our results provide the first data documenting de novo synthesis of the purine ring of theobromine. Additional experiments using the technique of isotope dilution to contrast the de novo synthesis of theobromine with its synthesis from exogenously supplied purine ribosides and bases provide evidence that the synthesis of theobromine occurs in two separate compartments in leaves of *C. arabica*, one utilizing purine rings generated endogenously and de novo, the other metabolizing exogenously supplied purines (Fig. 1). Taken together, the following results indicate that adenine and adenosine, when supplied exogenously, entered the purine pools of both compartments but did not move between compartments when produced endogenously: (a) unlabeled adenine and adenosine diluted the specific radioactivity of [^{14}C]formate incorporated into theobromine; (b) the specific radioactivity of [^{14}C]adenine or [^{14}C]adenosine incorporated into theobromine was diluted by exogenously supplied hypoxanthine, as well as other purine nucleotides and ribosides; (c) radiolabeled hypoxanthine was incorporated into ΣAde; and (d) unlabeled hypoxanthine did not dilute the specific radioactivity of [^{14}C]formate incorporated into theobromine. It is tempting to speculate that differences in the specificity of one or more membrane carrier protein provides the basis for the differential uptake between species of exogenously supplied purine ribosides and bases into the two compartments (pools). It is interesting to note that only adenine compounds have an amino group at position 6 of the purine ring, which could be the structural feature recognized by a specific carrier protein (Fig. 2).

The possibility that the de novo and salvage pathways occur in separate compartments (pools) between which transport of intermediates is slow was previously proposed to explain the unequal incorporation of precursors of the two pathways into adenine and guanine nucleotides (Shuster, 1963; Robern et al., 1965; Doree, 1973; Clark, 1974; Suzuki and Takahashi, 1975b, 1977; Anderson, 1979; reviewed by Ross, 1981). The physical location of the enzymes of the de novo purine pathway has been little studied (Wagner and Backer, 1992), with the exception of nitrogen-fixing nodules of the ureide-forming species *Vigna unguiculata* and *Glycine max*, in which the de novo pathway is clearly segregated in the plastids (Boland et al., 1982; Boland and Schubert, 1983). Whether this is true for nonureide-forming tissues remains to be determined.

Our results are the first to demonstrate the involvement of two separate compartments (pools) in the biosynthesis of a purine alkaloid. It is not known which of several possible enzymes regulates export from the de novo pool and entry into the catabolic pool of *C. arabica*: (a) AMP deaminase, which controls adenylate catabolism in spinach leaves and *Catharanthus roseus* cells in suspension culture (Yoshino and Murakami, 1980; Yabuki and Ashihara, 1992); (b) IMP oxidoreductase, which regulates the formation of xanthine for ureide biosynthesis in nitrogen-fixing nodules of cowpea (Shelp and Atkins, 1983); or (c) nucleobase nonspecific 5′-nucleotidases, which generate ribosides for intracellular transport (Wagner and Backer, 1992).

Our results clearly indicate that separate compartments (pools) are not involved in the biosynthesis of caffeine by

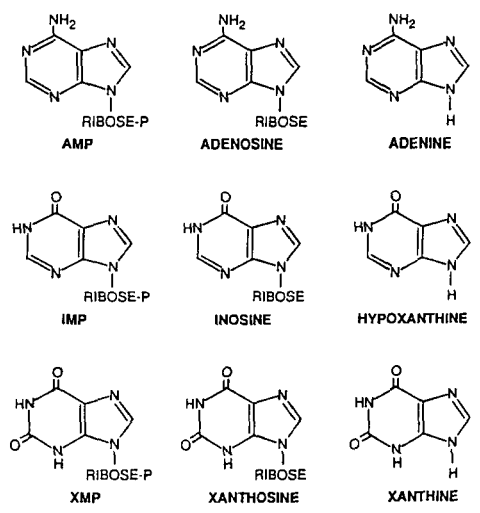

Figure 2. Structure of purine nucleotides, ribosides, and bases.

coffee leaves. Exogenously supplied XMP, inosine, hypoxanthine, and xanthine diluted the incorporation of either [^{14}C]-formate or [^{14}C]adenosine and [^{14}C]adenine into caffeine. The involvement of two separate pools in the biosynthesis of theobromine, but not caffeine, in leaves of *C. arabica* suggests that theobromine is not the immediate precursor of caffeine. This possibility is further substantiated by differences in the metabolism of xanthine: (a) [^{14}C]xanthine failed to label theobromine to a significant degree but labeled caffeine significantly; (b) unlabeled xanthine caused radiolabeled inosine, adenosine, adenine, and hypoxanthine to accumulate in theobromine but diluted the specific radioactivity of these compounds incorporated into caffeine (with the exception of inosine, the result of a single experiment, which we cannot explain at this time); and (c) allopurinol, a known inhibitor of the conversion of hypoxanthine to xanthine, caused radiolabeled adenosine and hypoxanthine to accumulate in theobromine but inhibited the incorporation of adenine and hypoxanthine into caffeine. The similarity in the results obtained with added xanthine versus allopurinol suggests the possibility that xanthine blocks the conversion of hypoxanthine to xanthine, as does allopurinol, resulting in the accumulation and storage of nitrogen and carbon in theobromine rather than in caffeine. Whether the reduced incorporation of radiolabeled purines into caffeine in the presence of added xanthine is due to isotope dilution or inhibition of hypoxanthine degradation remains to be determined.

Consistent with theobromine and caffeine being the products of two separate pathways, each was found to be preferentially synthesized from different monomethylated purine precursors. Although 7-methylxanthosine was only incorporated into theobromine in our system, previous researchers have provided evidence that 7-methylxanthosine was an efficient precursor of both theobromine and caffeine in leaves of both coffee (Baumann and Wanner, 1977) and tea (Negishi et al., 1985b). Of the dimethylpurines, paraxanthine was the best substrate for the synthesis of caffeine, as previously reported for both coffee fruits (Roberts and Waller, 1979) and leaves (Baumann and Wanner, 1977).

Reports in the literature defining a pathway for theobromine and caffeine biosynthesis are equivocal (Wagner and Backer, 1992). Our results provide strong evidence that theobromine is not the immediate precursor of caffeine and suggest that theobromine is synthesized from adenine nucleotides without a xanthine species as an intermediate, whereas caffeine is likely a product of a methylated xanthine. However, our results demonstrate that caffeine is synthesized from theobromine, and paraxanthine, when they are supplied exogenously. The results of our research underscore that a compound might be metabolized differently depending on whether it is supplied exogenously or generated endogenously, and we emphasize the need to understand these differences to elucidate the correct biosynthetic pathway. Thus, further investigation of the metabolism of endogenously generated purines is warranted to determine the exact enzymic events leading to the formation of each purine alkaloid.

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