PURINE METABOLISM AND ALKALOID BIOSYNTHESIS IN LEAVES OF *COFFEA ARABICA* L.

G. M. Nazario[1] and C. J. Lovatt[2]

[1]Department of Biology, University of Puerto Rico, PO BOX 23360, U.P.R. Station, San Juan P. R. 00931-3360; [2] Department of Botany and Plant Sciences, University of California, Riverside CA 92521-0124

ABSTRACT

The capacity of coffee (*Coffea arabica* L.) leaves to synthesize *de novo* and catabolize purine nucleotides to provide precursors for the synthesis of the purine alkaloid caffeine (1,3,7-trimethylxanthine) was investigated. Evidence that purine nucleotides and caffeine are synthesized *de novo* included: (i) incorporation of radiolabeled *de novo* precursors into IMP, the sum of adenine nucleotides, adenosine and adenine (∑Ade) and caffeine; (ii) confirmation that the radiolabel was incorporated into the purine ring of both IMP and adenine nucleotides; and (iii) inhibition of the incorporation of radiolabel into ∑Ade by the addition of azaserine, a known inhibitor of *de novo* purine synthesis in other organisms.

The conversion of radiolabeled purine ribosides and bases to xanthine provided evidence that degradation of purines in coffee leaves takes place via the classical pathway found in other organisms. Further evidence was provided by recovery of radiolabeled carbon 8 of hypoxanthine and xanthine in allantoin and recovery of radiolabeled carbon 8 of adenine, hypoxanthine and xanthine in urea.

Regulation of the activity of the *de novo* purine pathway by end-product inhibition was demonstrated for the first time in plants. Regulation of the pathway in *C. arabica* was contrasted with that of *Cucurbita pepo* , a species which does not synthesize purine alkaloids. The results indicated that the site of physiological importance in the regulation of the pathway by end-product inhibition in *C. arabica* was an enzymic step early in the *de novo* pathway, while end-product inhibition in *C. pepo* ocurred later in the *de novo* pathway at one or both of the enzymic reactions converting PRAICA to IMP.

Synthesis of the purine alkaloids, theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) in coffee leaves was demonstrated to utilize purines synthesized endogenously by the *de novo* pathway or supplied exogenously. The role of purines produced endogenously via the *de novo* pathway for the synthesis of these alkaloids was contrasted with that of purines generated via the various salvage or catabolic reactions from exogenously supplied pools. The specific question of whether theobromine or theophylline is the immediate precursor of caffeine was also addressed. The results provided strong evidence that unless supplied exogenously neither theobromine nor theophylline is the immediate precursor of caffeine and that each of the purine alkaloids is the product of a separate pathway. The data suggest that theobromine is synthesized from adenine nucleotides, theophylline from guanine nucleotides, and caffeine via the degradation of purines to xanthine with paraxanthine the immediate precursor of caffeine.

Although purine metabolism in higher plants has been demonstrated to be similar to that in bacteria and animals (6,9), plants producing unique purine alkaloids, such as caffeine (1,3,7-trimethylxanthine), may have alternative pathways for the synthesis, salvage or catabolism of purines.

During the investigation of caffeine biosynthesis we assessed the capacity of leaves of *Coffea arabica* to synthesize purine nucleotides *de novo*. In addition, we examined the subsequent metabolism of IMP to adenine nucleotides and the catabolism of the purine ring to xanthine and subsequently to urea. Since regulation of purine metabolism by end-product inhibition might be at a different enzymic step in a tissue producing purine alkaloids, we contrasted feedback control of purine *de novo* biosynthesis in leaves of *C. arabica* with that in excised roots of summer squash (*Cucurbita pepo*), a species which does not synthesize purine alkaloids.

Studies have been conducted with *Coffea arabica* (coffee) as well as with *Camellia sinensis*, to elucidate the pathway for the biosynthesis of caffeine. Anderson and Gibbs (1) and Proiser and Serenkov (7) demonstrated, respectively, that the purine ring of caffeine in coffee and in tea was synthesized from the same precursors utilized in the *de novo* biosynthesis of the purine ring, suggesting that the purine ring of caffeine is provided *de novo* . However, so far, most research attempting to elucidate the pathway of caffeine biosynthesis has been based on the salvage of exogenously supplied purine ribosides and bases. There is good evidence that exogenously supplied ribosides and bases are separately compartmentalized in plant cells from those generated endogenously (8). Thus, such research results may compromise attempts to identify the actual pathway of caffeine biosynthesis if it proceeds from purine rings generated endogenously.

Theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine) are the three methylated bases proposed as immediate precursors and/or catabolic products of caffeine. The bulk of the literature suggests that theobromine is the immediate precursor of caffeine, while theophylline has been proposed as a product of caffeine degradation (2). Very little work has been done with paraxanthine. The involvement of these xanthines in the synthesis of caffeine is still not conclusive.

In this study, we addressed the specific question of whether theobromine or theophylline is the immediate precursor of caffeine in coffee leaves.

MATERIALS AND METHODS

Plant material

Coffee (*Coffea arabica*) seedlings were donated from the Department of Agriculture in Puerto Rico and imported to California in accordance to USDA regulations. All experiments employed young fully expanded leaves from trees that were 1 to 5 years-old. The leaves were collected just prior to the experiments, washed with distilled water, and quickly blotted dry before weighing.

Seeds of summer squash (*Cucurbita pepo*) courtesy of Harris-Moran, were imbibed in distilled H_2O at room temperature for 24 hours. After germinating for two days, the roots were excised, pooled and immediately weighed into 150mg-aliquots which were maintained between sheets of moistened paper towel until weighing was completed (6).

Experimental

Incorporation of radiolabeled precursors into acid-soluble purine products: Conditions employed for the incorporation of [^{14}C]labeled substrates of purine metabolism by plant tissues were essentially those described by Lovatt (6).

Isolation of radiolabeled purine nucleotide products by cocrystallization: IMP or adenine nucleotides plus 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^{0}C for 1h prior to neutralization with KOH. Radiolabeled hypoxanthine and adenine synthesized by the plant tissue were isolated by cocrystallization with carrier hypoxanthine and adenine (6,9).

Isolation of radiolabeled PRAICA by TLC: PRAICA synthesized by leaf or root tissue was isolated from the neutralized acid soluble fraction by TLC on cellulose sheets using two different solvent systems as described by Ciardi and Anderson (4).

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 (5).

Isolation of radiolabeled theobromine, theophylline and caffeine by cocrystallization: Total theobromine, theophylline and caffeine were isolated from either acid-soluble or ethanol-soluble fraction with their respective carrier .

Results and Discussion

<u>*De novo* biosynthesis of purine nucleotides in leaves of *Coffea arabica* :</u> Evidence of the *de novo* pathway was provided by demonstration of the incorporation of the radiolabeled carbons of formate, bicarbonate and glycine into IMP and into ∑Ade (sum of the adenosine nucleotides, adenosine and adenine) (Table 1). Inosine monophosphate (IMP) was subjected to acid hydrolysis and isolated as hypoxanthine. In addition, adenine nucleotides and adenosine synthesized during the incubations were subjected to acid hydrolysis and isolated as adenine, confirming that the labeled carbons were in both cases incorporated into the purine ring.

Table 1: Incorporation of [^{14}C]labeled precursors of the *de novo* purine biosynthetic pathway into IMP and into the sum of adenine nucleotides, adenosine and adenine (∑Ade) in intact cells of *Coffea arabica.*

Conditions for the incorporation of 100 mM [^{14}C]glycine (400 dpm/nmol) were optimal; no attempts were made to determine optimal conditions for 25 mM [^{14}C]bicarbonate (2,640 dpm/nmol) or 5 mM [^{14}C]formate (3,300 dpm/nmol).

[^{14}C]-Precursor	nmol precursor incorporated into product per g fr wt leaf tissue during a 3 h incubation	
	IMP	∑Ade
Glycine	25 ± 5[a]	37 ± 5
Bicarbonate	4 ± 0	42 ± 2
Formate	8 ± 4	61 ± 7

[a] Mean ± standard error, *n* = 2.

In addition, azaserine, a known inhibitor of*de novo* purine biosynthesis which is effective in plants (6,9), at concentrations of 5-20 mM inhibited by about 60% the incorporation of [^{14}C]bicarbonate into ∑Ade in leaves *C. arabica* .

<u>Catabolism of purines in leaves of *C. arabica*:</u> Evidence of the catabolic pathway was provided by the incorporation of both [^{14}C]formate and [^{14}C]adenosine into hypoxanthine: 10 and 1306 nmol, respectively, incorporated into hypoxanthine per g fresh weight leaf tissue during a 7-h incubation period. The radiolabeled ribosides inosine and adenosine and the bases adenine and hypoxanthine were all converted to xanthine (Table 2). The incorporation of these radiolabeled precursors was inhibited in the presence of allopurinol (10 mM final concentration), a known inhibitor of the enzymes which catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid (3) (Table 2).

Table 2. Incorporation of radiolabeled inosine, adenosine, adenine and hypoxanthine into xanthine by leaves of *Coffea arabica* incubated in the presence of allopurinol (10 mM final concentration).

No attempts were made to determine optimal conditions for the incorporation of 2 mM [^{14}C]inosine (1,100 dpm/nmol), 2 mM [^{14}C]adenosine (1,100 dpm/nmol), 2mM [^{14}C]adenine (1,100 dpm/nmol) or 2mM [^{14}C]hypoxanthine (200 dpm/nmol). Xanthine was isolated from the neutralized acid soluble fraction by co-crystallization with carrier xanthine.

[^{14}C]labeled Precursor	Incorporation of precursor into xanthine[a]	
	Control	+ allopurinol (10 mM)
	nmol/g fresh wt · 7h	% control
Inosine	341	57
Adenosine	3030	75
Adenine	3264	15
Hypoxanthine	3890	44

[a] Mean ± SE (*n*=2-3)

Furthermore, 7, 113 and 316 nmol of [^{14}C]urea were formed from [8-^{14}C]adenine, [8-^{14}C]hypoxanthine and [8-^{14}C]xanthine, respectively, per g fr wt leaf tissue during a 3-h incubation period. Urease activity was demonstrated by the release of 120 ± 9 nmol (*n*=2) $^{14}CO_2$ from exogenously supplied [^{14}C]urea per g fr wt leaf tissue during a 3-h incubation period.

Regulation of the *de novo* purine biosynthetic pathway in *Coffea arabica* contrasted with regulation of the *de novo* purine pathway in *Cucurbita pepo*: The effect of added adenosine on the activity of the *de novo* purine pathway of *C. arabica* was contrasted with *C. pepo* , a species which does not synthesize purine alkaloids. Adenosine or its metabolites inhibited the incorporation of radiolabeled bicarbonate and formate into IMP and ∑Ade by 90%. In the presence of added adenosine, [^{14}C]bicarbonate and [1-^{14}C]glycine, but not formate, accumulated in PRAICA (5'-phosphoribosyl-4-carboxamide-5-aminoimidazole). These results suggest that end product inhibition in *C. pepo* occurs at one or both of the enzymic reactions converting PRAICA to IMP.

In contrast, for *C. arabica* , adenosine or its metabolites inhibited the incorporation of [^{14}C]bicarbonate into PRAICA, IMP and ∑Ade. 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]glycine into RAICA.

We were unable to identify the specific site of regulation for the *de novo* purine pathway of coffee, but results suggest a difference in the site of physiological importance in the regulation of the *de novo* pathway of *C. pepo* vs *C. arabica.*

Evidence that theobromine, theophylline and caffeine are the products of separate pathways in leaves of *Coffea arabica* : The effect of added XMP, inosine, adenosine, adenine, hypoxanthine, xanthine and guanosine on the incorporation of [^{14}C]formate into theobromine, theophylline and caffeine suggested potential differences in the metabolic pathway leading to the synthesis of the three purine alkaloids. Incorporation of radiolabeled formate was reduced in the presence of exogenously supplied adenosine and adenine, while XMP, inosine, hypoxanthine and xanthine failed to reduced the incorporation. Incorporation of [^{14}C]formate into theophylline was reduced in the presence of XMP, inosine, adenosine, adenine and guanosine, but not in the presence of hypoxanthine or xanthine. On the other hand, the presence of exogenously supplied XMP, inosine, adenosine, adenine, hypoxanthine and xanthine reduced the specific radioactivity of [^{14}C]formate incorporated into caffeine (Scheme 1).

Formate-----------> IMP----------->**Theobromine**

Additive	% inhibition
adenosine	52
adenine	81

Formate-----------> IMP----------->**Theophylline**

Additive	% inhibition
XMP	28
Inosine	51
Adenosine	65
Adenine	74
Guanosine	85

Formate-----------> IMP-----------> **Caffeine**

Additive	% inhibition
XMP	55
Inosine	10
Adenosine	53
Adenine	50
Hypoxanthine	51
Xanthine	30
Guanosine	30

Scheme 1. Effect of unlabeled purine additives on the incorporation of radiolabeled formate into theobromine, theophylline and caffeine.

Coffee leaves metabolized xanthine into theobromine in a different manner than in caffeine. We obtained no significant incorporation of [^{14}C]xanthine into theobromine. However, unlabeled xanthine (10 mM final concentration) increased the incorporation of radiolabeled ribosides and bases into theobromine. Allopurinol either increased or had no effect on the incorporation of these precursors into

theobromine. In contrast, 90 ± 22 (n=3) nmol [^{14}C]xanthine were incorporated into caffeine per g fr wt coffee leaves during a 7-h incubation period. Consistent with xanthine being an intermediate in the synthesis of caffeine, unlabeled xanthine (10 mM final concentration) reduced the specific radioactivity of radiolabeled formate, adenosine, adenine and hypoxanthine incorporated into caffeine.

In regards to theophylline and caffeine the most striking differences in the metabolism of formate were in the presence of added hypoxanthine and xanthine, which caused significant accumulation of [^{14}C]formate in theophylline, but reduced the specific radioactivity of [^{14}C]formate incorporated into caffeine.

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 provided evidence that the synthesis of theobromine occurs in two separate compartments in leaves of *Coffea arabica*, one utilizing purine rings generated endogenously and *de novo*, and the other metabolizing exogenously supplied purines. Results suggest that adenine and adenosine, when supplied exogenously, entered the purine pools of both compartments, but did not move between compartments when produced endogenously (Figure 1).

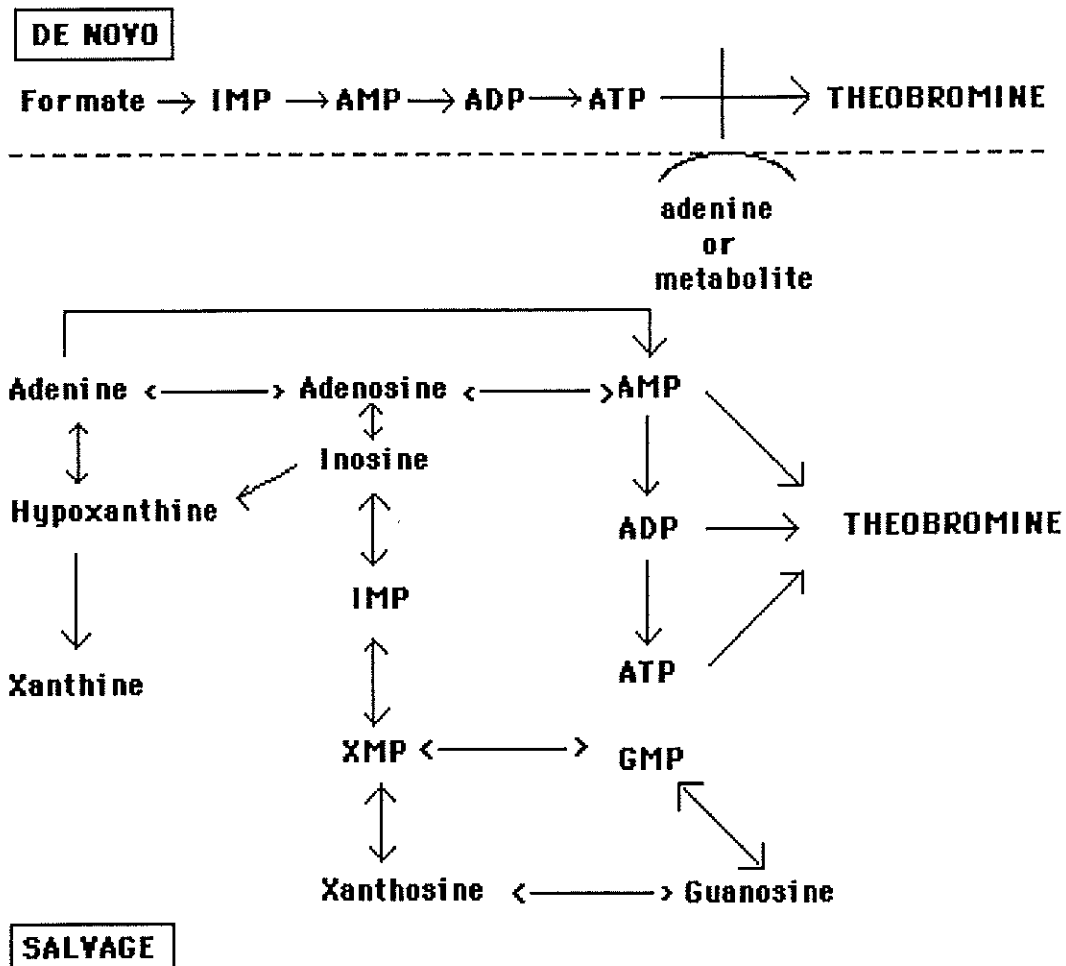

Figure 1. Compartmentalization of the *de novo* and salvage pathways in the synthesis of theobromine in leaves of *Coffea arabica.*

SUMMARY

The occurrence of the classical pathway for the *de novo* biosynthesis of purine nucleotides was suggested by demonstration of the incorporation of several [^{14}C]precursors into IMP and ∑Ade and by the inhibition of this incorporation by azaserine, a known inhibitor of *de novo* purine biosynthesis in other organisms.

Evidence demonstrating purine degradation via the classical pathway was provided by incorporation of the radiolabeled purine ribosides inosine and adenosine and the radiolabeled bases adenine and hypoxanthine into xanthine. In addition, allopurinol, a known inhibitor of xanthine oxidase and xanthine dehydrogenase, in all cases inhibited the incorporation of radiolabeled precursors into xanthine. Coffee leaves degraded 316 nmoles of [8-^{14}]xanthine to urea per g fresh weight in 3-h confirming that urea is a product of degradation.

Incorporation of radiolabeled bicarbonate into IMP and ∑Ade was inhibited by the addition of adenosine to excised roots of *C. pepo* and leaves of *C. arabica* suggesting that the purine *de novo* pathway was regulated by end-product inhibition in both species. Regulation by end-product inhibition in *C. pepo* , a species which does not synthesize caffeine, was different from regulation in *C. arabica* . Although we were unable to identify the specific site of the *de novo* purine pathway sensitive to end-product inhibition in coffee, results suggest that an early step in the pathway prior to the synthesis of PRAICA is the site of physiological importance in the regulation of the pathway. In contrast, the site of physiological importance in the regulation of the *de novo* purine pathway in squash was at one or both of the enzymic reactions converting PRAICA to IMP.

The pathway for the biosynthesis of theobromine, theophylline and caffeine was also investigasted. Theobromine, theophylline and paraxanthine are logical precursors of caffeine. However, our results provided strong evidence that theobromine, theophylline and caffeine are the products of different pathways. The results suggested that theobromine is synthesized from adenine nucleotides, theophylline from guanine nucleotides and caffeine from the degradation of purines via hypoxanthine to xanthine with paraxanthine the immediate precursor of caffeine.

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