Biosynthesis of Caffeine in Leaves of Coffee

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The levels of endogenous caffeine and theobromine were much higher in buds and young leaves of Coffea arabica L. cv Kent than in fully developed leaves. Biosynthesis of caffeine from 14C-labeled adenine, guanine, xanthosine, and theobromine was observed, whereas other studies (H. Ashihara, A.M. Monteiro, T. Muritz, F.M. Gillies, A. Crozier [1996] Planta 198: 334–339) have indicated that there is no detectable incorporation of label into caffeine when theophylline and xanthine are used as substrates for in vivo feeds with leaves of C. arabica. The capacity for caffeine biosynthesis, especially from guanine and xanthosine, was reduced markedly in both fully developed mature and aged leaves. Data obtained in pulse-chase experiments with young leaves indicate the operation of an AMP → IMP → xanthosine 5'-monophosphate (or GMP → guanosine) → xanthosine → 7-methylxanthosine → 7-methyltheobromine → theobromine → caffeine pathway. The data obtained provide strong evidence against proposals by G.M. Nazario and C.J. Lovatt ([1993] Plant Physiol 103: 1203–1210) concerning the independence of caffeine and theobromine biosynthesis pathways and the role of xanthine as a key intermediate in caffeine biosynthesis.

Caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) have been found in more than 60 subtropical plant species (Suzuki et al., 1992). Many plants used for nonalcoholic beverages, such as tea (Camellia sinensis L.), coffee (Coffea arabica L.), cocoa (Theobroma cacao), and maté (Ilex paraguariensis), contain these purine alkaloids (Suzuki and Waller, 1988). The biosynthesis pathway of theobromine and caffeine has been the subject of much study over the years. Although early investigations implied the involvement of nucleic acids as precursors in caffeine biosynthesis (Ogutuga and Northcote, 1970; Suzuki and Takahashi, 1976), more recent investigations with tea and coffee suggest that caffeine is produced from the purine nucleotides AMP, GMP, and/or IMP and that theobromine is the immediate precursor of caffeine (Fig. 1) (Negishi et al., 1985, 1992; Fujimori et al., 1990; Suzuki et al., 1992; Fujimori and Ashihara, 1994). In coffee, young leaves that are not fully expanded have the highest capacity for caffeine biosynthesis (Fujimori and Ashihara, 1994).

On the basis of metabolic studies in which the identification of products and the level of incorporation of 14C label were based on co-crystallization, Nazario and Lovatt (1993a, 1993b) concluded that theobromine is not the immediate precursor of caffeine in coffee leaves and that two separate de novo and salvage pools are involved in the biosynthesis of theobromine. Despite an absence of confirmatory analytical evidence, the Nazario and Lovatt papers raise serious questions concerning the validity of the caffeine biosynthesis pathway illustrated in Figure 1. In these circumstances, there is an urgent need for clarification. The current report describes an investigation of caffeine biosynthesis in coffee leaves in which metabolites originating from radiolabeled [8-14C]adenine, [8-14C]guanine, [8-14C]xanthosine, and [2-14C]theobromine were analyzed by HPLC-RC and TLC. The data obtained with these labeled purine bases and nucleosides enabled the proposals of Nazario and Lovatt (1993a, 1993b) to be examined in detail.

MATERIALS AND METHODS

Plant Material

Leaves were obtained in July and August 1994 from 4-year-old coffee plants (Coffea arabica L. cv Kent) growing under a natural photoperiod in a greenhouse at the University of Glasgow. The developmental stages of the leaves were categorized as (a) buds and young leaves, (b) mature leaves, and (c) aged leaves. Young leaves were the most recently emerged, and they weighed approximately 25 mg (fresh weight) and were approximately 20 mm long and 7 mm wide. Mature leaves comprised the fully expanded, second and third leaves below the apex (weight approximately 1.2 g), whereas aged leaves were dark green near the base of the shoot and weighed approximately 1.3 g.

Chemicals

The following radiochemicals were purchased from the commercial sources indicated: [8-14C]adenine (specific activity 1.96 MBq μmol⁻¹), Amersham International; [8-14C]guanine (2.1 MBq μmol⁻¹) Amersham; [2-14C]theobromine (2.07 MBq μmol⁻¹), Moravek Biochemicals (Brea, CA); and [8-14C]xanthosine (2.07 MBq μmol⁻¹), Moravek. Radiolabeled uric acid, allantoin, and allantoic acid, used for co-chromatography, were prepared in vitro from

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Figure 1. Metabolic pathways illustrating both the biosynthesis of caffeine from purine nucleotides and the catabolism of purine nucleotides to CO₂ and NH₃ in leaves of C. arabica. XMP, Xanthosine 5'-monophosphate.

[2-¹⁴C]xanthine (1.94 MBq μmol⁻¹) using xanthine oxidase (buttermilk), uricase (Candida utilis), and allantoinase (peanut). These enzymes and all other chemicals were purchased from Sigma.

Extraction of Endogenous Xanthine Derivatives

Segments of coffee leaves were boiled in water for 15 min and homogenized using a pestle and mortar. The resulting homogenate was centrifuged at 12,000g for 10 min, after which the supernatant and pellet were removed and replaced with fresh medium without the radiolabeled substrate.

Metabolism of Radiolabeled Purine Derivatives

Segments of C. arabica leaves (5 × 5 mm for mature and aged leaves; 5-mm strips of buds and young leaves) were incubated in 2 mL of medium, comprising 30 mM potassium phosphate buffer, pH 5.6, 10 mM Suc, and a radiolabeled substrate, contained in a 30-mL Erlenmeyer flask, in a shaking water bath at 27°C. The Erlenmeyer flask had a well center containing a small glass tube into which was inserted a piece of filter paper wetted with 0.1 mL of a 20% potassium hydroxide solution. In pulse-chase experiments, after an appropriate period, the incubation medium was removed and replaced with fresh medium without the radiolabeled substrate.

At the end of the incubation period the glass tube and filter paper from the center well were transferred to a 50-mL flask containing 10 mL of distilled water, and after thorough shaking, radioactivity in a 1-mL aliquot was determined by liquid scintillation counting to estimate the amount of ¹⁴CO₂ released during the metabolism period. The C. arabica leaf segments were separated from the incubation medium by filtering through a tea strainer, washed with 50 mL of distilled water, then mixed with 2 mL of extraction medium, comprising 80% methanol in 20 mM sodium diethylidithiocarbamate, and ground in a chilled pestle and mortar. The resultant tissue homogenate was centrifuged at 12,000g for 5 min, and the supernatant and pellet were separated. The pellet was resuspended in 5 mL of extraction medium and re-centrifuged. After three extractions, the supernatant fractions containing the methanol-soluble metabolites were combined to yield a total volume of approximately 12 mL, which was reduced to dryness in vacuo, and aliquots were analyzed by liquid scintillation counting, HPLC-RC, and/or TLC. The pellet was treated with 4 mL of 6% perchloric acid at 100°C for 20 min to hydrolyze nucleic acids, after which the sample was centrifuged at 12,000g for 5 min, and the pellet was washed with cold 6% perchloric acid prior to re-centrifugation and a further extraction. The three supernatant fractions were combined and a 20-μL aliquot was removed for estimation of radioactivity by liquid scintillation counting.

HPLC Analysis of Endogenous Xanthine Derivatives and Radiolabeled Metabolites

A Spectra Physics (San Jose, CA) 8700 liquid chromatograph was used to deliver a 25-min, 0 to 40% gradient of methanol in 50 mM sodium acetate, pH 5.0, at a flow rate of 1 mL min⁻¹, with samples being introduced off-column via a 7125 valve (Rheodyne, Cotati, CA) with a 500-μL loop. Reversed-phase HPLC utilized a 250 × 4.6-mm-i.d. universal ferruleless column (Capital HPLC Specialists, Broxburn, Lothian, UK), packed in-house with a 5-μm ODS Hypersil support (Shandon, Runcorn, Cheshire, UK). Column eluate was directed first to a Spectra Physics 8450 A270 monitor, after which fractions were collected or the solvent was mixed with liquid scintillant (10 g L⁻¹ 2,5-diphenyloxazole in Triton X-100/xylene/methanol [11:2.5, v/v/v]), pumped at a flow rate of 3 mL min⁻¹ via a Reeve Analytical (Glasgow, UK) 9702 reagent delivery unit, and directed to a Reeve Analytical 9701 radioactivity monitor with a 500-μL spiral glass flow cell. Signals from both detectors were processed by a dual-channel 27000 data-handling system (Reeve Analytical). The limit of detection for ¹⁴C-labeled compounds analyzed by reversed-phase HPLC-RC, operating in the homogeneous mode, is approximately 3 to 4 Bq (Sandberg et al., 1987).

The reversed-phase HPLC system successfully separated 11 different purine derivatives and also was able to resolve ¹⁴C-labeled uric acid, allantoin, and allantoic acid produced enzymatically from [8-¹⁴C]xanthine (Ashihara et al., 1996).

TLC Analysis of Radiolabeled Metabolites

Because nucleotides were retained on the reversed-phase HPLC column, methanol-soluble radiolabeled metabolites...
were also subjected to TLC. Methanolic extracts were subjected to TLC on 200- × 200-mm sheets of microcrystalline cellulose (Spotfilm; Tokyo Kasei Kogyo, Tokyo, Japan) using a solvent of n-butanol/acetic acid/water (4:1:2, v/v). Radiolabeled spots, detected after 2 weeks of exposure using Kodak X-OMAT AR film (Eastman Kodak), were scraped off the TLC plates and eluted from the cellulose support with water, and the radioactivity was measured by liquid scintillation counting (Ashihara and Kubota, 1986).

The recoveries of all 14C-labeled standards after HPLC and TLC were >90%; therefore, it was not necessary to apply a correction factor to adjust the data obtained for sample losses.

RESULTS

Levels of Endogenous Xanthine Derivatives

Only caffeine, theobromine, and xanthine were present in detectable quantities when leaf and bud extracts were analyzed by HPLC (Table I). Buds and young leaves contained the highest concentration of caffeine with about one-third as much being detected in mature and aged leaves. Theobromine and xanthine were present in much lower concentrations than caffeine, and the levels of theobromine, in particular, were markedly reduced in mature and aged leaves. Theophylline (1,3-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine) were not detected in any of the leaf extracts.

Metabolism of [8-14C]Adenine

Figure 2 illustrates the results of pulse-chase experiments with [8-14C]adenine and young leaves of C. arabica. Adenine nucleotides, nucleic acids, and theobromine were the most heavily labeled compounds after the 6-h pulse. The radioactivity associated with these compounds, as well as 7-methylxanthosine and 7-methylxanthine, declined after the leaves were transferred to a nonradioactive medium. In contrast, 14C-labeled caffeine and CO2 increased after the pulse period. The accumulation of [14C]caffeine in this manner is in keeping with the fact that the alkaloid is synthesized much more rapidly than it is catabolized in C. arabica shoots (Ashihara et al., 1996). The data presented in Table II, which were obtained after an 18-h incubation with [8-14C]adenine, indicate that the capacity of leaves to convert adenine into theobromine and caffeine is highest in young leaves and decreases as the leaves mature and age.

Figure 2. Distribution of radioactivity in metabolites from [8-14C]adenine after a pulse-chase experiment with young leaves of C. arabica. Leaves (50 mg fresh weight) were incubated with 9.5 µM [8-14C]adenine for 6 h, after which the radioactivity was chased for a further 66 h. Incorporation of radioactivity is expressed as a percentage of total radioactivity taken up by the leaves (7.1 ± 0.3 kBq 50 mg−1 fresh weight). □, Adenine nucleotides; ●, theobromine; ○, caffeine; △, 7-methylxanthosine; ▲, 7-methylxanthine; ◆, CO2; ■, RNA. Similar data were obtained in a duplicate experiment.

Table II. Overall metabolism of [8-14C]adenine by young, mature, and aged leaves of C. arabica

Leaf samples were incubated with 9.4 µM [8-14C]adenine (1.96 MBq µmol−1) for 18 h. Total uptake of radioactivity is expressed as kBq 100 mg−1 of leaf (fresh weight), means ± SE (n = 3). Incorporation of radioactivity into metabolites is expressed as a percentage of total uptake ± SE (n = 3). n.d., Not detected.
Metabolism of [8-14C]Guanine

Figure 4 illustrates the analysis of methanol-soluble metabolites of [8-14C]guanine by HPLC-RC. In addition to the guanine substrate, the radiolabeled compounds detected included caffeine, theobromine, 7-methylxanthine, allantoin, and allantoic acid. The incorporation of radioactivity into theobromine and caffeine was higher in young than in mature and aged leaves (Fig. 4; Table III). This may be due partly to a decrease in guanine salvage activity as the leaf matures and ages. The decrease in the incorporation of radioactivity from [8-14C]guanine into nucleic acids observed in the older leaves supports this possibility. In contrast, degradation of [8-14C]guanine, as indicated by the release of 14CO2, increased during leaf development (Table III), and this was accompanied by an increase in the level of radioactivity associated with the purine catabolites allantoin and allantoic acid (Fig. 4). Most of these degradation products appear to be produced directly from guanine by conventional purine catabolism pathways, independently of the production of purine alkaloids (Fig. 1).

Metabolism of [8-14C]Xanthosine

Figure 5 illustrates the HPLC-RC profiles obtained with the methanol-soluble fractions obtained from leaves of C. arabica after a 1-h incubation with [8-14C]xanthosine. Xanthosine is an intermediate of purine catabolism (Fig. 1; Suzuki et al., 1992), and as a consequence, much of the exogenously supplied substrate was probably degraded via this route and incorporation of radioactivity into caffeine was detected only in young leaves. In keeping with its role in purine catabolism, with all three types of leaf, most of the applied [8-14C]xanthosine was converted to allantoin and allantoic acid.

To further examine the caffeine biosynthesis pathway from [8-14C]xanthosine, pulse-chase experiments were carried out with young leaves in the presence and absence of 5 mM allopurinol (Table IV). In control incubations 7-methylxanthine was the major radioactive product, accounting for 87% of the total radioactivity in young leaves. In contrast, when 5 mM allopurinol was present, caffeine and theobromine were produced in young leaves, accounting for 80% of the total radioactivity. The decrease in the incorporation of [8-14C]adenine in caffeine.

Table III. Overall metabolism of [8-14C]guanine by young, mature, and aged leaves of C. arabica

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Young</th>
<th>Mature</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residual guanine</td>
<td>4.0 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>7-Methylxanthine</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Theobromine</td>
<td>11.7 ± 1.8</td>
<td>1.0 ± 0.2</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Caffeine</td>
<td>9.1 ± 0.5</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Theophylline</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Other soluble compounds</td>
<td>3.4 ± 1.0</td>
<td>6.4 ± 1.9</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>CO2</td>
<td>35.4 ± 5.2</td>
<td>62.6 ± 4.0</td>
<td>74.6 ± 1.7</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>35.6 ± 0.7</td>
<td>25.6 ± 2.1</td>
<td>18.9 ± 1.6</td>
</tr>
<tr>
<td>Total uptake of radioactivity</td>
<td>24.7 ± 1.0</td>
<td>26.7 ± 3.6</td>
<td>29.9 ± 1.6</td>
</tr>
</tbody>
</table>
ylxanthine and theobromine were both labeled during the 6-h pulse, but no radioactivity was associated with these compounds after an 18-h chase. Some of the substrate was converted to caffeine, but the majority appeared to undergo degradation via the purine catabolism pathway with incorporation of label into allantoin, allantoic acid, and CO₂ (Table IV). Inhibition of the purine catabolism with allopurinol resulted in a marked reduction in 14CO₂ output and an absence of detectable incorporation of label into allantoin and allantoic acid, whereas there was an increase in radioactivity associated with xanthine, 7-methylxanthine, and caffeine (Table IV).

Although Nazario and Lovatt (1993a, 1993b) reported that coffee leaves convert 8-14C]xanthine to caffeine, Ashihara et al. (1996) demonstrated that 2-14C]xanthine is incorporated into allantoin, allantoic acid, and CO₂, but not caffeine, by young, mature, and aged C. arabica leaves, even in presence of allopurinol. The metabolism of 8-14C]xanthosine to caffeine, demonstrated in Figure 5 and Table IV, is therefore in keeping with the operation of a xanthosine pathway (Fig. 1). Radioactivity was associated with all of the intermediates in this pathway except 7-methylxanthosine, which presumably was subject to rapid turnover, being metabolized to 7-methylxanthine at a rate that precluded its accumulation in detectable quantities.

**Metabolism of [2-14C]Theobromine**

The metabolism profile of the methanol-soluble fraction obtained after feeding 2-14C]theobromine revealed that theobromine is converted exclusively to caffeine by young, mature, and aged leaves after an 18-h incubation period (Fig. 6). The total amount of 14C]caffeine to accumulate was 1611 Bq (100%) in young leaves, 1064 Bq (66%) in mature leaves, and 614 Bq (33%) in aged leaves. This implies that the capacity of leaves to carry out this 1-N-methylation step does not decline with age to the same extent as other sections of the caffeine biosynthesis pathway, as indicated by the level of conversion of radiolabeled adenine, guanine, and xanthosine to caffeine in young, mature, and aged leaves (Tables II and III; Fig. 5).

**DISCUSSION**

There is strong evidence that the biosynthesis of caffeine is initiated by the degradation of purine nucleotides (Ashihara and Kubota, 1986, 1987; Fujimori et al., 1991; Negishi et al., 1992). In higher plants, as well as almost all other organisms, purine nucleotides are synthesized by both de novo and salvage pathways (Ross, 1981; Hirose and Ashihara, 1984; Wagner and Backer, 1992). The metabolic fate of purine nucleotides is usually investigated using radiolabeled purine bases and nucleosides (Yabuki and Ashihara, 1991). Since adenine is not readily degraded in higher plants because they lack adenine deaminase (Le Floc’h et al., 1982), it is relatively easy to follow the metabolic fate of adenine nucleotides that have been prelabeled with 8-14C]adenine. Although guanine can be degraded by guanine deaminase, the activity of this enzyme in plants is low (Negishi et al., 1994); therefore, exogenous 8-14C]guanine is usually converted to 8-14C]GMP in significant amounts by hypoxanthine-guanine phosphoribosyltransferase (Hirose and Ashihara, 1984).

In the present study, adenine and guanine nucleotides were prelabeled by feeding 8-14C]adenine and 8-14C]guanine, and the biosynthesis of caffeine from these purine nucleotides was investigated. Data obtained in pulse-chase experiments with 8-14C]adenine (Fig. 2; Table II) suggest strongly that caffeine is synthesized from adenine nucleotides via 7-methylxanthosine, 7-methylxanthine, and theobromine, as illustrated in Figure 1. The demonstrated conversions of 8-14C]xanthosine to 7-methylxanthine, theobromine, and caffeine (Fig. 5; Table IV) and that of 2-14C]theobromine to caffeine (Fig. 6) provide convincing evidence for the operation of this pathway. These findings clearly refute the proposal of Nazario and Lovatt (1993a, 1993b) that theobromine is not an immediate precursor of caffeine in C. arabica leaves. Nazario and Lovatt (1993a, 1993b) presented data indicating that, although allopurinol inhibited the conversion of 8-14C]adenine to caffeine by more than 50%, it had no effect on the incorporation of label into theobromine. This implies that the biosynthesis of caffeine from adenine involves reactions catalyzed by xanthine dehydrogenase/oxidase, and it was concluded that caffeine is synthesized from the degradation of purines via hypoxanthine, xanthine, and paraxanthine, whereas theobromine is produced from adenine nucleotides (Nazario and Lovatt, 1993a, 1993b). The data presented in this report do not confirm these proposals. In our experiments, although 5 mM allopurinol strongly inhibited purine catab-
olism, it also brought about small increases in the incorporation of \([8-^{14}C]\)adenine into both theobromine and caffeine (Fig. 3). This was probably due to an increase in the size of the adenine nucleotide pool facilitated by the inhibitory effects of allopurinol on purine catabolism (Fig. 1).

Biosynthesis of caffeine from \([8-^{14}C]\)guanine in *C. arabica* leaves was also demonstrated in the current studies. The relatively high level of accumulation of \([^{14}C]\)caffeine in young coffee leaves (Fig. 5A) implies that the applied guanine is converted to GMP (Fig. 1). Since GMP reductase is not functional in plant cells (Ashihara et al., 1992), 5’-nucleotidase and guanosine deaminase (Negishi et al., 1994) are probably responsible for the metabolism of GMP to guanosine and on to xanthosine, which is incorporated into the caffeine biosynthesis pathway, as indicated in studies with tea leaves (Suzuki et al., 1992). Although \([8-^{14}C]\)guanine was clearly converted to theobromine and caffeine, no incorporation of label into either theophylline or paraxanthine was observed (Fig. 5; Table III). This finding does not support the hypothesis of Nazario and Lovatt (1993a) that theophylline is synthesized from guanine nucleotides.

The data obtained in the present study demonstrate that there is a marked decline in the rate of biosynthesis of caffeine from adenine, guanine, and xanthosine as *C. arabica* leaves age, but because caffeine is catabolized very slowly (Ashihara et al., 1996), there is not a concomitant reduction in the concentration of endogenous caffeine (Table I). The reduced rate of incorporation into caffeine is probably an indirect consequence of enhanced purine catabolism in the mature and aged leaves, which restricts the availability of xanthosine as a substrate for caffeine biosynthesis (Fig. 1). In contrast to adenine, guanine, and adenosine, the rate of conversion of theobromine to caffeine is not influenced greatly by leaf age (Fig. 6). Whether other committed steps in the caffeine biosynthesis pathway behave in a similar manner remains to be determined.

The proposed role of xanthine as a key intermediate in the biosynthesis of caffeine by Nazario and Lovatt (1993a, 1993b) is especially controversial because previous in vivo and in vitro studies with both tea and coffee have indicated that conversion of xanthine to caffeine is at best negligible (Looser et al., 1974; Suzuki and Takahashi, 1975b) and, more usually, undetectable (Suzuki and Takahashi, 1975a; Roberts and Waller, 1979; Suzuki and Waller, 1984; Negishi et al., 1985). Reviewing the available evidence in 1992, Suzuki et al. concluded that xanthine cannot serve effectively as the direct precursor of the caffeine ring, and recent studies in which \([2-^{14}C]\)xanthine was incubated with young, mature, and aged *C. arabica* leaves did not detect incorporation of label into caffeine (Ashihara et al., 1996).

The major discrepancies between our findings and the data of Nazario and Lovatt (1993a, 1993b) may result from...
the use of different plant material and experimental methods. Our data were obtained mainly with very young leaves of C. arabica, which have a high capacity for caffeine biosynthesis, and overall metabolism was examined by TLC and high-resolution HPLC-RC after the application of leaves of C. arabica, which have a high capacity for caffeine biosynthesis in coffee plants. J Biol Chem 237: 1941-1944


Nazario GM, Lovatt CJ (1993b) Separate de novo and salvage purine pools are involved in the biosynthesis of theobromine but not caffeine in leaves of Coffea arabica L. Plant Physiol 103: 1203-1210


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