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N-METHYLTRANSFERASES AND 7-METHYL-N^9-NUCLEOSIDE HYDROLASE ACTIVITY IN *COFFEA ARABICA* AND THE BIOSYNTHESIS OF CAFFEINE*

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Key Word Index—*Coffea arabica*; Rubiaceae; biosynthesis; caffeine; theobromine; theophylline; paraxanthine; enzymology.

Abstract—The incorporation of radioactivity from L-[$^{14}CH_3$]-methionine into caffeine by coffee fruits was enhanced by additions of theobromine and paraxanthine but was reduced by additions of theophylline and caffeine. Cell-free extracts prepared from seedlings, partially ripe and unripe coffee fruits showed that only the unripe green fruits contained significant methyltransferase and 7-methyl-N^9-nucleoside hydrolase activity. The cell-free extracts catalysed the transfer of methyl groups from *S*-adenosyl-L-[$^{14}CH_3$]-methionine to 7-methylxanthine, and 7-methylxanthosine, producing theobromine and to theobromine producing caffeine. The two enzymic methylations exhibited a sharp pH max at 8.5 and a similar pattern of effects with metal chelators, thiol reagents and Mg^{2+} ions, which were slightly stimulating though not essential to enzyme activity. Paraxanthine (1,7-dimethylxanthine) was shown to be the most active among methylxanthines as methyl acceptors; however its formation from 1-methylxanthine and 7-methylxanthine was not detectable, and biosynthesis from paraxanthine in the intact plant would therefore appear not to occur. The apparent K_m values are as follows: 7-methylxanthine 0.2 mM, theobromine 0.2 mM, paraxanthine 0.07 mM and *S*-adenosyl-L-methionine with each substrate 0.01 mM. The results suggest the pathway for caffeine biosynthesis in *Coffea arabica* is: 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is synthesized from the same precursors utilized for purine and methyl group synthesis in other systems [1–3]. Among the methylated xanthines, 7-methylxanthine and theobromine (3,7-dimethylxanthine) have been reported as precursors of caffeine [4, 5]. However, the pathway leading to 7-methylxanthine is less obvious, but recent work [5,6] strongly supports the view that ring formation in caffeine follows the classical scheme for purine nucleotide biosynthesis [7]. As compared to intact plants and callus tissue from coffee and tea plants, cell-free systems are advantageous for study since the direct proof for the involvement of *S*-adenosyl-L-methionine and the sequence of methylations of the purine ring can only be obtained by the use of such systems. Recent work with cell-free extracts from tea leaves proves that *S*-adenosyl-L-methionine functions there as methyl donor and that the biosynthesis of caffeine occurs via 7-methylxanthine and theobromine [8]. Attempts by Suzuki and Takahashi [8] to prepare active cell-free preparations from coffee leaves were unsuccessful. The present work shows that active cell-free extracts may be prepared from coffee fruits and the *S*-adenosyl-L-methionine *N*-methyltransferase and *N*-methyl-N^9-nucleoside hydrolase activity of these preparations has been investigated and compared with the activity of the tea leaf extracts.

RESULTS AND DISCUSSION

L-*Methionine incorporation into detached coffee fruits*

Earlier work [9] has shown that the caffeine content of coffee fruits rapidly increases in seeds of age 2.5–4 months whereas the caffeine content of the pericarp remains appreciably unaltered over the period 0–7 months. The incorporation of L-[$^{14}CH_3$]-methionine into the pericarp caffeine was found to be 25 times that into the seed caffeine. Caffeine synthesis appeared to be considerably enhanced by light.

In the experiments recorded in Table 1, L-[$^{14}CH_3$]-methionine was fed to detached coffee fruits of varying age. (1. Kew, approximately 7–8 month old; 2. Kew, approximately 2–3 month old; 3. Kings College, approximately 2–3 month old.) In each instance appropriate substrates were also added to the solutions fed to each fruit. The results show that the methyl group of L-methionine is actively incorporated into the caffeine of coffee fruits during the ripening process. However, in the older fruits of sample 1, additions of theobromine and paraxanthine show enhancement of caffeine formation, suggesting a shortage of dimethylated xanthine substrates at this stage in development, whereas addi-

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Table 1. Incorporation of L-[$^{14}CH_3$]-methionine by detached fruits of *Coffea arabica*

Additions	Hours before harvesting	% Incorporation of L-[$^{14}CH_3$]-methionine into caffeine		
		1	2	3
No addition	14	—	4.95 ± 0.98	—
+ theobromine	,,	—	9.45 ± 0.68	—
+ theophylline	,,	—	—	—
+ paraxanthine	,,	—	19.9*	—
+ caffeine	,,	—	—	—
No addition	24	1.38 ± 0.41	6.35 ± 1.02	20.5 ± 3.8
+ theobromine	,,	0.28 ± 0.01	15.2 ± 1.98	8.7 ± 0.7
+ theophylline	,,	0.18 ± 0.09	—	—
+ paraxanthine	,,	n.d.	13.6 ± 4.22	20.5 ± 2.8
+ caffeine	,,	0.33 ± 0.09		
No addition	43–48	1.96 ± 0.08	—	22.5 ± 5.8
+ theobromine	,,	5.25 ± 2.6	—	18.0 ± 2.0
+ theophylline	,,	0.44 ± 0.03	—	—
+ paraxanthine	,,	—	—	36.5 ± 2.2
+ caffeine	,,	0.85 ± 0.01	—	—

1 = Red fruits (Kew Gardens); 2 = green fruits (Kew Gardens); 3 = green fruits (Kings College). Each result is the average of duplicate results with detached fruits of average dry weight for —1 (220 mg); 2 (220 mg); 3 (40 mg).

Each fruit fed L-[$^{14}CH_3$]-methionine; 1 (5.0 μmol = 4.4×10^6 dpm av. uptake 99%); 2 (2.5 μmol = 2.0×10^{-6} dpm av. uptake 98%); 3 (3.2 μmol = 2.8×10^6 dpm av. uptake 90%).

The amount of methylxanthine added during feeding of L-[$^{14}CH_3$]-methionine to each fruit was: 1800 μg; 2, 300 μg; 3, 300 μg.

* Only one experiment was conducted.

tions of caffeine and theophylline appear to be slightly inhibitory. It is, however, possible that increased amounts of caffeine stimulate the formation of other metabolites from caffeine, and both possibilities require further investigation. In the young fruits (samples 2 and 3) theobromine is readily available and additions appear to reduce caffeine formation; however, there is a location difference and a varietal difference which apparently affect the concentrations of theobromine in the fruits. The results generally show theobromine and paraxanthine to be equally good substrates for caffeine formation. Since the degree to which individual coffee fruits took up the precursor and the rate at which it was metabolized within the fruit were both very variable, subsequent investigations of caffeine formation were made using cell-free extracts. These were prepared from coffee fruits of samples 2 and 3 since the results of the feeding experiments clearly suggest that these fruits are metabolically more active in the formation of caffeine than the older fruits. Indeed the change in caffeine content of fruits from 0 to 7 months [9] would indicate that fruits at 3–4 months old suddenly increase their production of caffeine in the seed although levels of the alkaloid in the pericarp remain constant. This suggested also that methyl-transferase activity was more active at this stage in the development of the fruit; this is borne out by our subsequent measurement of the methyltransferase activity of cell-free extracts of coffee fruits.

Extraction of N-*methyltransferase*

The preparation of active cell-free extracts was only possible with the use of Polyclar AT and rigorous exclusion of oxygen as described in the Experimental. It was also essential to remove phenolic materials rapidly by gel filtration. The protein content of the resulting extracts was low (3 mg/ml) and this in part was responsible for the extreme lability of the methyltransferase. Similar

Table 2. The formation of caffeine from dimethylxanthines by cell-free extracts of coffee seedlings and berries using *S*-adenosyl-L-[$^{14}CH_3$]methionine as methyl donor

	Theobromine	Theophylline	Paraxanthine (cpm)
Coffee seedlings	—	—	—
Green coffee berries	5223	190	7235
Red/yellow partially ripe coffee berries	36	7	154

The complete system contained substrate (0.5 mg/ml) 30 μl; $MgCl_2$ (3 mM) 10 μl; *S*-adenosyl-L-[$^{14}CH_3$]methionine 1.4 mCi/mmol) 20 μl (final concn 1.4×10^{-4} M) enzyme solution (62 μg protein) 20 μl; 50 mM Tris buffer pH 8.5 to 100 μl. Samples without $MgCl_2$ showed a 10% reduction in activity. Control sample either with boiled enzyme or minus the substrate had no detectable activity. Incubation was for 60 min at 30°.

preparations were made with young coffee seedlings but in these the formation of caffeine was not detectable and only very low activity was present in partially ripe fruits.

N-Methyltransferase activity in cell-free preparations was detected by using *S*-adenosyl-L-[$^{14}CH_3$]-methionine as the methyl donor and the dimethylxanthines, theobromine, theophylline and paraxanthine, as acceptor since all three can be utilized in caffeine formation [7]. Table 2 shows that only cell-free extracts from the young green coffee fruits contained significant activity, as was expected from the incorporation of L-methionine by the whole fruits. Assays in which boiled protein solution or no protein were used showed no activity. The results given in subsequent sections are from at least three separate experiments with duplicates. Two preparations were made from the green coffee fruits, the interval between the preparations being three months. These gave similar results. A combination of TLC and radiography was used for the separation and identification of the ^{14}C-labelled methylxanthines. After incubation with the enzyme solution it was shown that the radioactivity transferred from *S*-adenosyl-L-[$^{14}CH_3$]-methionine occurred only in the appropriate product (depending on the substrate used) and that this activity was only slightly increased by Mg^{2+} (10%).

Properties of the coffee berry methyltransferase catalysing caffeine synthesis

The effect of time and enzyme concentration. The rates of formation of theobromine and caffeine from 7-methylxanthine and theobromine respectively were found to be linear with time up to at least 7 min and was proportional to the amount of enzyme solution added (up to at least 186 μg protein per assay) under the conditions of the standard assay described in the Experimental.

Stability of methyltransferase activity. The enzyme extracts lost 50% of their activity over a period of 1 hr at 4°, though this rapid loss in activity could be reduced if enzyme solutions were fortified with bovine serum albumin protein (BSA) 3 mg/ml. It was found however, that enzyme solutions stored in liquid N_2 with or without the BSA fortification and with BSA addition would withstand 3–4 thawings before significant (50%) loss of activity was observed. It was therefore possible to carry out most of the experiments on methyltransferase activity on the same preparation. However, the green fruits harvested and immediately frozen with solid CO_2 kept well at −20° and subsequent preparations with these fruits made over a period of six months showed similar activity. It was thus possible to harvest fruits at a time of maximum methyltransferase activity and have active material available over a period of time far in excess of the short natural period of high methyltransferase activity.

pH Profile of methyltransferase activity. The pH optimum in 50 mM Tris Buffer at 30° appeared sharply at 8.5 with both 7-methylxanthine and theobromine as substrate. These results are similar to those given for theobromine and caffeine formation with cell-free extracts from tea leaves [8] except that the pH curve for the conversion of theobromine to caffeine exhibited a broader maximum with tea cell-free extracts.

Effects of inhibitors on methyltransferase activity. Table 3 illustrates the action of inhibitors on the enzyme activity. Significant inhibition by thiol reagents was observed, *p*-chloromercurobenzoic acid (*p*CMB) being the most effective. Some inhibition was observed with NaN_3 (10 mM) and KCN (100 mM). However, KCN (10 mM) whilst inhibitory for the methylation of 7-methylxanthine, appeared slightly to stimulate the methylation of theobromine. Some slight inhibition was also observed with azouracil. EDTA had no effect on activity.

Substrate specificity of methyltransferase. To learn whether the pathways of caffeine biosynthesis in coffee fruits were similar to those found in tea leaves [7], the further methylation of a series of methylxanthines was

Table 3. The effect of inhibitors on methyltransferase activity in green coffee berries

Concentration of inhibitor (mM)	% Activity 7-Methylxanthine	Theobromine
No inhibitor	100	100
p-CMB (0.5)	0	0
(0.05)	70	78
Iodoacetate (100)	0	0
(10)	21	11
NaN_3 (10)	61	78
(1)	90	84
KCN (100)	12	26
(10)	56	133
Azouracil (1)	72	100
(0.1)	80	82
N-methylmaleimide (1)	0	0
(0.1)	83	100
EDTA (5)	94	100
(0.5)	100	100

Coffee berry enzyme extract (20 μl 62 μg protein) was pre-incubated 5 min at 300° with the complete incubation mix minus *S*-adenosyl-L-methionine plus the inhibitor at the concentrations indicated and then the activity was measured under standard assay conditions.

Table 4. Substrate specificity of methyltransferase

Substrate (mM)		Methylated product	n-Katals	Relative methylation %
7-Methylxanthine	1.5	Theobromine	7.6	79
3-Methylxanthine	1.5	—	—	—
1-Methylxanthine	1.5	Theophylline	133	4.5
Theobromine	1.5	Caffeine	6.2	100
Theophylline	1.5	Caffeine	169	3.6
Paraxanthine	1.5	Caffeine	4.0	138
Xanthine	1.5	—		—
Hypoxanthine	1.5	—	—	—
Xanthosine	1.5	—	—	—
7-Methylxanthosine	1.5	Theobromine	99.0	6.1

The complete system contained substrate (0.5 mg/ml) 50 µl $MgCl_2$ (3 mM) 10 µl; *S*-adenosyl-L-[$^{14}CH_3$]methionine (1.4 Ci/mmol) 20 µl (final concn 1.4×10^{-4} M) enzyme solution (62 µg protein) 20 µl 50 mM Tris buffer pH 8.5 to 100 µl. Incubation was at 30° for 10 min. A unit of protein is taken as 1 mg. With the substrates xanthine, hypoxanthine and xanthosine incubation was for 60 min.

investigated. The results in Table 4 show that 1-methylxanthine produced theophylline and 7-methylxanthine produced only theobromine. As with tea leaf extracts [7], paraxanthine was the most active of the dimethylxanthines, the formation of caffeine being 1.5 times that for the formation of caffeine from theobromine. However, the formation of paraxanthine from 1-methylxanthine was low and that from 7-methylxanthine was not detectable, so that paraxanthine is probably not a normal precursor of caffeine *in vivo*. The synthesis of theophylline from 1-methylxanthine is slow, as is the synthesis of caffeine from theophylline. Unlike the situation with tea leaf extracts [7], the synthesis of theobromine from 7-methylxanthine is lower than the synthesis of caffeine from theobromine.

Investigation of the utilization of xanthine, hypoxanthine, xanthosine and 7-methylxanthosine showed that of these substrates only 7-methylxanthosine could act as a substrate for theobromine formation with coffee fruit cell-free extracts, thus indicating the presence of an active 7-methyl-N^9-nucleoside hydrolase. Like the leaf disc experiments of Looser *et al.* [5], these results would support 'Pathway II' of coffee biosynthesis proposed by Ogutuga and Northcote [4], in which methylation occurs at N-7 of guanine in a polynucleotide and subsequently 7-methylguanylic acid is transformed via 7-methylguanosine to 7-methylxanthosine, which is converted via a 7-methyl-N^9 nucleoside hydrolase to 7-methylxanthine and subsequently to caffeine (Fig. 1). Furthermore, the fact that theophylline in coffee cell-free extracts is formed from 1-methylxanthine rather than 3-methylxanthine corresponds with the findings with tea cell-free extracts [5], and thus substantiates the view [5] that the biosynthesis of theophylline from xanthine via 3-methylxanthine is unlikely [4].

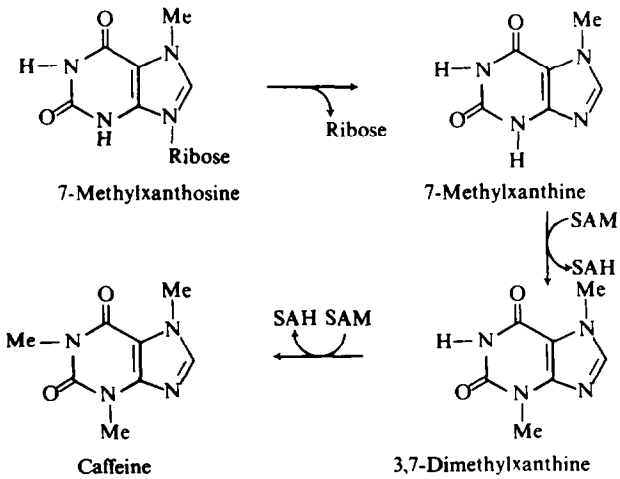

Fig. 1. Formation of caffeine from 7-methylxanthosine in *Coffea arabica*. SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine.

Effect of substrate concentration on methyltransferase activity. The rate of production of theobromine and caffeine was measured at various concentrations of 7-methylxanthine, theobromine, paraxanthine and *S*-adenosyl-L-methionine. The rate of production of theobromine at various concentrations of 7-methylxanthine and *S*-adenosyl-L-methionine were determined from the Lineweaver–Burk plot. The K_m values for 7-methylxanthine and *S*-adenosyl-L-methionine were 0.2 and 0.01 mM, respectively. Similarly the K_m values for theobromine and *S*-adenosyl-L-methionine were 0.2 and 0.01 mM respectively, and for paraxanthine and *S*-adenosyl-L-methionine 0.07 and 0.01 mM.

These results differ significantly from those recorded for tea cell-free extracts [8] in that the concentration requirement for the methylated xanthine substrate is lower by a factor of 10. However, as with tea methyltransferase activity, estimations of the apparent K_m values are the same for theobromine and caffeine formation, although these could change with further purification. The two reactions have similar pH optima and there is little difference in their behaviour with inhibitors; hence the question of whether one or two separate enzymes are involved in caffeine synthesis remains unsolved. Although the preliminary feeding experiments suggested that theobromine might be an inhibitor at high concentration and thus regulate the formation of caffeine, increasing the concentration of this substrate by a factor of 100 had no inhibitory effect in the cell-free extracts. Similarly additions of caffeine to a concentration of 10 mM had no effect on caffeine formation. It must be assumed therefore that these methylxanthines inhibit protein synthesis rather than enzyme activity.

EXPERIMENTAL

Plant material. Unripe coffee fruits at various stages from petal fall, were obtained from King's College, University of London, and the Royal Botanic Gardens, Kew. Experiments in which L-[$^{14}CH_3$]-methionine was fed to detached coffee fruits were performed *in situ* and material for the preparation of cell-free systems was collected and transported packed in solid CO_2. These berries were stored at $-20°$ until required.

Feeding experiments with L-[$^{14}CH_3$]*-methionine and detached coffee fruits.* Fruits were removed from the branches of coffee trees by cutting under H_2O. The fruits were then placed immediately in Eppendorf tubes with their petioles immersed in distilled H_2O or L-[$^{14}CH_3$]-methionine (1–2 μCi, specific activity 56 mCi/mmol) or L-[$^{14}CH_3$]-methionine plus either theobromine, theophylline, paraxanthine or caffeine (300–800 μg). The coffee fruit samples used were (1) green/yellow fruits approximately 5–6 months old; (2) red/yellow fruits 7–8 months old; both samples from Kew Gardens, and (3) green fruits approximately 2 months old from Kings College, London University. Duplicate samples were harvested over a period of 14–48 hr and the samples immediately frozen at $-79°$ (dry ice) and stored in a freezer at $-20°$. Experiments showed no loss of caffeine on vacuum drying at 50° and therefore these samples were dried in this manner before further processing at Oklahoma State University. The caffeine was isolated using the methods of Frischnecht *et al.* [10] and TLC, and radioactivity measurements were made essentially according to Looser *et al.* [5].

Preparation of cell-free systems from green coffee fruits. Fourteen green coffee fruits (11–12 g) were pulverized in a mortar in the presence of solid CO_2 and liquid N_2. The resulting powder was rapidly added to previously hydrated Polyclar AT and stirred in an atmosphere of N_2 for 30 min. The Polyclar AT was hydrated by allowing it to stand 20 min in 35 ml of extracting buffer I (200M phosphate pH 7.3, 5% ascorbate, 5 mM 2-mercaptoethanol and 5 mM EDTA). The Polyclar AT was subsequently removed by squeezing the slurry through cheesecloth followed by centrifugation at 38000 *g* for 15 min. The supernatant (25 ml) was placed on a Sephadex G25 column (1.5 × 30 cm) and eluted with buffer II (50 mM phosphate pH 7.3, 2 mM 2-mercaptoethanol; 2 mM EDTA). The first 20 ml of protein solution were collected for use as the enzyme preparation.

Assay for N*-methyltransferase activity.* Enzyme activity was measured using an assay based on the transfer of a ^{14}C-labelled methyl group from *S*-adenosyl-L-methionine (SAM) to the appropriate xanthine to produce theophylline, theobromine and caffeine. The standard assay mixture contained substrate (1 mg/ml) 30 μl; $MgCl_2$ (3 mM) 10 μl; [$^{14}CH_3$]-SAM (1.45 mCi/mmol) 20 μl (final concn 1.4×10^{-5} M) enzyme inhibitor (if any) 10 μl; enzyme soln 20 μl and 50 mM Tris buffer pH 8.5 to 100 μl. The reaction was carried out in Eppendorf tubes with pre-incubation at 30° for 5 min. The reaction was initiated with the enzyme and terminated with 10% HCl (200 μl) after an incubation period of 10 min at 30°. Controls consisted of samples with boiled enzyme or minus enzyme.

Isolation of the reaction products. The xanthines were extracted from the acidified incubation mix with $CHCl_3$ (1 ml + 2 × 0.5 ml). The appropriate carrier xanthine (5 μg) was added and the solutions taken to dryness. The xanthines were separated using TLC on prepared Al backed Si Gel G/F_{450} plates; solvent system $CHCl_3$–MeOH (9:1); visualization was by UV. The appropriate xanthine product was cut out from the TLC plate and placed in a scintillation vial with EtOH 1 ml and 10 ml of scintillation fluid (P.P.O. 12.5 g dimethyl/POPOP 0.75 g in toluene 2.5 l). The radioactivity was determined and hence the amount of product formed could be deduced.

Determination of protein. This was determined using the method of Lowry *et al.* [11]. A unit of protein was taken as 1 mg.

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