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CAFFEINE BIOSYNTHESIS STARTS WITH THE METABOLICALLY CHANNELLED FORMATION OF 7-METHYL-XMP—A NEW HYPOTHESIS

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Key Word Index—*Coffea arabica*; Rubiaceae; caffeine biosynthesis; 7-methylxanthosine; 7-methyl-XMP; purine alkaloids; purine metabolism.

Abstract—Young coffee leaflets were fed with [U-^{14}C]adenine of [*Me*-^{14}C]methionine, and the incorporation of radioactivity into purines (purine bases, nucleosides, nucleotides, nucleic acids) and purine alkaloids (PA) (7-methylxanthine, theobromine, caffeine) was determined after 4 and 24 hr. Labelling of PA was considerable after 4 hr (i.e. about 38 or 71% of the recovered radioactivity from [U-^{14}C]adenine and [*Me*-^{14}C]methionine, respectively) and, on an absolute basis, distinctly higher after 24 hr signifying a continuous synthesis and precursor supply in the isolated leaflets. Despite these favourable conditions and the most selective approach to methyl group labelling, i.e. use of [*Me*-^{14}C] methionine, we were not able to show the incorporation of radioactivity into either 7-methylxanthosine, the formerly postulated key compound at the onset of PA synthesis, or any monomethylated purine other than 7-methylxanthine. The same negative result was obtained in our recent studies with coffee cell suspension cultures and implies that the first methylation step in caffeine synthesis is metabolically channelled. Additionally, enzyme extracts were prepared from the same tissue and methyltransferase activity was measured using various substrates. Among them only xanthosine and XMP were methylated, and exclusively at the N-7 position of the purine ring. XMP *N*-methyltransferase activity, which had not been detected before, was measured under conditions which strongly reduced hydrolysis of both educt and product (e.g. by Na_2MO_4 as inhibitor of nucleotidase activities). These results, together with our recent studies, allow us to present a novel hypothesis regarding caffeine biosynthesis which favours *methylation* of XMP rather than xanthosine as the crucial step leading from primary metabolism into caffeine synthesis. However, this first methylation is considered to be metabolically channelled.

INTRODUCTION

Ten years ago Negishi *et al.* [1–3] postulated the SAM-dependent methylation of xanthosine as the key reaction connecting primary metabolism with caffeine biosynthesis. The resulting product, 7-methylxanthosine, had already been postulated by earlier workers as an intermediate yielding 7-methylxanthine upon de-ribosylation [4–7]. The two subsequent SAM-mediated methylations of the latter to theobromine and caffeine, respectively, were the first-known steps of purine alkaloid (PA) synthesis. They have been confirmed by various investigators (for a review see ref. [8]), whereas the *in situ* methylation of xanthosine has only been reported by the Japanese researchers mentioned above.

In a recent study, we re-examined the initial methylation step, and by the use of suspension-cultured cells of *Coffea arabica* and a wide range of experimental conditions [9, 10] we came to the conclusion that methylation of xanthosine occurs, if at all, in a metabolic channel. This, however, would imply two separate xanthosine pools, as presented in Scheme 1 of ref. [10]. In summary, the experimental evidence was as follows: (1) no significant *in situ* formation of [^{14}C]7-methylxanthosine could be shown after both [U-^{14}C]adenine and [*Me*-^{14}C]methionine feeding, although xanthosine (after [^{14}C]adenine feeding) as well as the purine alkaloids (PA) 7-methylxanthine, theobromine, and caffeine exhibited considerable radiolabelling, in particular after stimulation of caffeine biosynthesis by 'cold' adenine and ethephon; (2) the specific activity of xanthosine was lower than that of 7-methylxanthine and in no case showed the time course characteristics of a precursor.

This and the observation that enzyme extracts of the same source contained remarkable nucleotidase activities against 7-methyl-XMP and 7-methyl-GMP [10] led us to reconsider methylation at the nucleotide level as the very onset of caffeine biosynthesis, although previous attempts to demonstrate nucleotide *N*-methyltransferase activity had failed [2, 11].

Therefore, and to meet the justified criticism that tissue cultures are not representative of the *in situ* situation, we

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(a) re-examined the formation of monomethylated nucleotide or nucleoside caffeine precursors from [U-^{14}C]adenine and [*Me*-^{14}C]methionine using coffee leaflets, and (b) thoroughly tested enzyme extracts from the same foliar tissue for their ability to methylate at the N-7 position of the purine ring.

RESULTS AND DISCUSSION

Metabolism of [^{14}*C*]*adenine*

When coffee leaflets were fed with [^{14}C]adenine, 39 and 78% was taken up after 4 and 24 hr, respectively. The related incorporation into purine metabolites is presented in Table 1. After 4 hr, 38% of the recovered radioactivity was already distributed among the purine alkaloids, namely 7-methylxanthine (13%), theobromine (18%) and caffeine (7%). Although the total activity in the PA still increased between 4 and 24 hr, the relative incorporation remained fairly constant (39% after 24 hr), whereas labelling of 7-methylxanthine (6% after 24 hr) decreased in favour of theobromine (26% after 24 hr). Similarly, the percentage incorporation into nucleic acids remained at the constant level of 29%. Acid hydrolysis of the nucleic acid fraction (see experimental) revealed only [^{14}C]adenine and [^{14}C]guanine, the ratio (adenine: guanine) of 19:1 and 7:1 after 4 and 24 hr, respectively. The relative as well as the absolute incorporation into the nucleotide fraction (only AMP, ADP, and ATP were found to be labelled) decreased during the experiment, this was in contrast to purine alkaloids and nucleic acids which showed, as indicated above, a continuous labelling. There was a very conspicuous increase of radioactivity in adenine, i.e. from 2 to 17% (or ×25 on an absolute basis) between 4 and 24 hr, most likely as the result of RNA turnover. Little incorporation was also observed in SAM and NAD. However, in contrast to the [^{14}C]adenine feeding experiments with coffee cell suspension cultures [10], radioactivity was not present in any of the nucleosides (i.e. adenosine, inosine, guanosine, xanthosine) nor were the purine catabolites allantoin and uric acid labelled.

Table 1. Incorporation of [U-^{14}C]adenine into purine metabolites by young coffee leaflets after 4 and 24 hr

	4 hr		24 hr	
Metabolites	(nCi)	(%)	(nCi)	(%)
7-Methylxanthine*	31.6	12.7	34.4	5.8
Theobromine*	45.3	18.2	151.8	25.5
Caffeine*	16.9	6.8	45.9	7.7
Σ Purine alkaloids*	93.7	37.7	232.1	39.0
ATP	29.6	11.9	25.6	4.3
ADP	6.7	2.7	10.9	1.8
AMP	26.5	10.7	15.2	2.6
Σ Nucleotides	62.7	25.3	51.7	8.8
SAM	16.4	6.6	16.4	2.6
NAD	0	0	16.5	2.8
Adenine	4.0	1.7	102.7	17.3
Nucleic acids	56.3	28.7	137.5	28.9
Total radioactivity	233.2	100	560.4	100

Leaflets (*ca* 300 mg fr. wt) were incubated with 2 ml medium containing 1.5 μCi (2.8 μM) [U-^{14}C]adenine. After 4 and 24 hr *ca* 150 mg of the leaflets were harvested, extracted and analysed by HPLC and on-line radiodetection.

*Includes [U-^{14}C]purine alkaloids which diffused into the incubation buffer.

Table 2. Incorporation of [Me-^{14}C]methionine into methylated compounds by young coffee leaflets after 4 and 24 hr

	4 hr		24 hr	
Metabolites	(nCi)	(%)	(nCi)	(%)
7-Methylxanthine*	55.3	23.1	86.2	20.2
Theobromine*	47.0	19.7	66.9	15.7
Caffeine*	67.4	28.2	74.5	17.5
Σ Purine alkaloids*	169.8	70.9	227.7	53.3
SAM	11.9	5.0	0	0
Methionine sulphoxide	45.3	18.9	127.5	29.9
Methionine	12.4	5.2	71.8	16.8
Total radioactivity	239.3	100	427.0	100

Leaflets (*ca* 300 mg fr. wt) were incubated with 1 ml medium containing 4 μCi (88.8 μM) [*Me*-^{14}C]methionine. After 4 and 24 hr *ca* 150 mg of the leaflets were harvested, extracted and analysed by HPLC and on-line radiodetection.

*Includes the [U-^{14}C]purine alkaloids which diffused into the incubation buffer.

Finally, neither [^{14}C]7-methylxanthosine nor any other radiolabelled N-7-methylated compound besides PA could be detected in this study with leaflets, this corresponding essentially to the [^{14}C]adenine feeding experiments with coffee cell suspension cultures [10].

Metabolism of [Me-^{14}*C*]*methionine*

The rate of uptake of [*Me*-^{14}C]methionine was low as compared to [^{14}C]adenine and amounted to 7 and 29% after 4 and 24 hr, respectively. As shown in Table 2, more than 70% of the recovered radioactivity was distributed between 7-methylxanthine (23%), theobromine (20%) and caffeine (28%) after 4 hr. Obviously, the rate of purine alkaloid synthesis slowed down between 4 and 24 hr and a considerable amount of the [^{14}C]methionine was oxidized to [^{14}C]methionine sulphoxide. [Me-^{14}C]methionine was also incorporated into SAM (5% after 4 hr), but no radioactivity could be detected in the nucleic acid fraction, 7-methylxanthosine or in any other compound, this again is in accordance with feeding experiments with coffee cell suspension cultures [10].

Studies with enzyme extracts

Conventional assay for N-*methyltransferases.* As a first approach we used assay conditions similar to those

Table 3. Methylation of purine metabolites by enzyme extracts of young coffee leaflets (conventional assay)

Substrate (0.5 mM)	Methylated products	Relative methylation (%)	
Theobromine	Caffeine		47
7-Methylxanthine	Theobromine		100
Xanthosine	7-Methylxanthosine	21	
	7-Methylxanthine	52	73
XMP	7-Methylxanthosine	20	
	7-Methylxanthine	48	68
Guanosine	—		—
GMP	—		—
Inosine	—		—
IMP	—		—
Adenosine	—		—
AMP	—		—
Xanthine	3-Methylxanthine		15

The incubation mixture contained 20 μl 0.5 M tricine–NaOH buffer (pH 7.5), 50 μl of enzyme extract from coffee leaflets, 10 μl 10 mM $MgCl_2$, 10 μl (0.05 μCi) of *S*-adenosyl-L-[*Me*-^{14}C]methionine (60 mCi mmol^{-1}) and 10 μl of the substrate (methyl acceptor) at the concentration listed. The incubation was carried out at RT and stopped after 60 min by adding 10 μl 1.2 M $HClO_4$. The reaction mixture was analysed by HPLC and on-line radiodetection.

Table 4. Methylation of purine metabolites by enzyme extracts of young coffee leaflets (modified assay)

Substrate (mM)	Methylated products	Relative methylation (%)	
7-Methylxanthine (0.5)	Theobromine		100
XMP (0.5)	7-Methyl-XMP	4	
	7-Methylxanthosine	59	
	7-Methylxanthine	20	83
XMP (5.0)	7-Methyl-XMP	10	
	7-Methylxanthosine	67	
	7-Methylxanthine	27	104
XMP (5.0)	7-Methyl-XMP	16	
+ Na_2MO_4 (1.0)	7-Methylxanthosine	52	
	7-Methylxanthine	23	91

The incubation mixture contained 20 μl 0.5 M tricine–NaOH buffer (pH 7.5), 50 μl enzyme extract from coffee leaflets, 10 μl 10 mM $MgCl_2$, 10 μl (0.05 μCi) *S*-adenosyl-L-[*Me*-^{14}C]methionine (60 mCi mmol^{-1}) and 10 μl of the substrate (methyl acceptor) and nucleotidase inhibitor (Na_2MO_4) at the concentration listed. The incubation was carried out at RT and stopped after 20 min by heating at 150° for 45 s. Immediately before stopping the reaction 10 μl 5 mM 7-methyl-XMP was added to the assay. The reaction mixture was analysed by HPLC and on-line radiodetection.

described for the determination of theobromine *N*-methyltransferase, 7-methylxanthine *N*-methyltransferase and xanthosine *N*-methyltransferase [2, 7, 11]. Desalted enzyme extracts of young coffee leaflets were incubated with *S*-adenosyl-L-[*Me*-^{14}C]methionine (8.4 μM) as the methyl donor and a purine (0.5 mM) as the methyl acceptor. After incubation for 1 hr, [^{14}C]methylated as well as unlabelled products were analysed by HPLC with on-line radiodetection.

As shown in Table 3, xanthosine was an excellent substrate for enzymic methylation at the N-7 position of the purine ring. However, 7-methylxanthine was also formed in this assay, most likely as the result of 7-methylxanthosine nucleosidase activity, since 7-methylxanthine cannot originate directly from the xanthine formed to a very small extent (only traces after 1 hr) by the action of xanthosine nucleosidase. As shown in a separate assay (Table 3), xanthine was exclusively methylated at N-3.

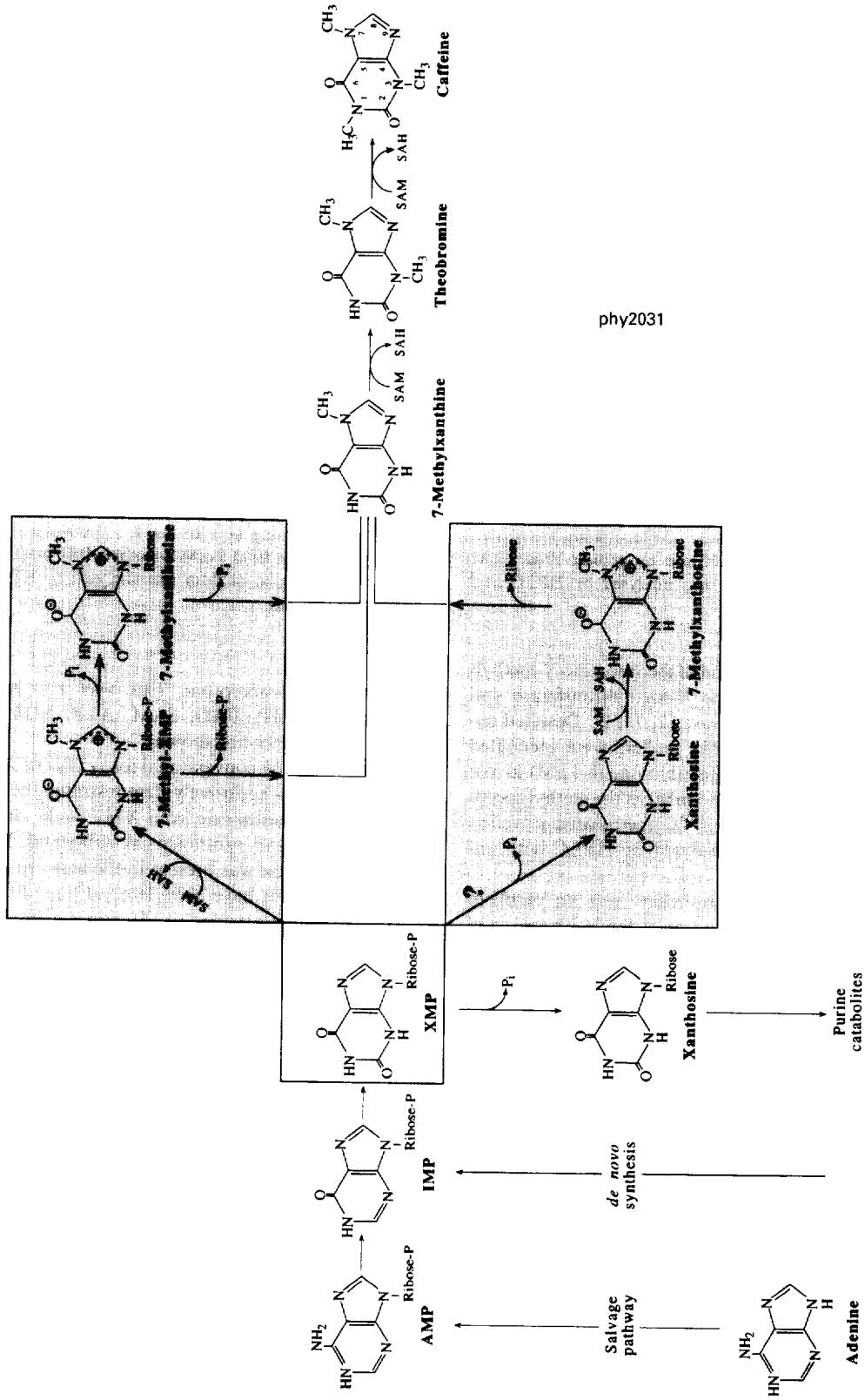
When XMP served as substrate, no 7-methyl-XMP but only 7-methylxanthosine and 7-methylxanthine were detected as [^{14}C]methylated products. At first sight this suggested that XMP was methylated and subsequently hydrolysed to 7-methylxanthosine and 7-methylxanthine. However, analysis of the unlabelled products showed that more than 90% of the XMP was hydrolysed to xanthosine by XMP nucleotidase within the incubation time of 1 hr. Thus, the methylated products were more likely to have been formed by methylation of xanthosine followed by hydrolysis by 7-methylxanthosine nucleosidase rather than by methylation of XMP.

All of the other substrates tested (GMP, IMP, AMP, guanosine, inosine, adenosine; see Table 3) were inactive as methyl group acceptors. This was also true for NAD(H), NADP(H), FAD, SAM, ATP, ADP, GTP, GDP and IDP (data not shown).

In conclusion, the conventional assay (Table 3) for *N*-methyltransferases exclusively demonstrates the formation of 7-methylxanthosine from XMP (only after nucleotidase action) and xanthosine as substrates. If XMP *N*-methyltransferase was present in the assay mixture, it would probably not have been detected due to the high nucleotidase activity towards XMP and 7-methyl-XMP. Furthermore, considerable hydrolysis is likely to occur as a result of the addition of $HClO_4$ to terminate the reaction.

Modified assay for XMP N-*methyltransferase.* In order to improve the search for the related activity and to prevent as much as possible the reactions covering up the formation of 7-methyl-XMP, the assay conditions were changed as follows: (1) the XMP concentration was increased to 5 mM, (2) nucleotidases were inhibited by addition of 1 mM Na_2MO_4, (3) the reaction was stopped after 20 min by heating at 150° for 45 s, and (4) 7-methyl-XMP (0.5 mM) was added immediately before heating.

As shown in Table 4, the formation of [^{14}C]7-methyl-XMP was observed under all conditions tested, and was experimentally increased from 4% at 0.5 mM XMP to10 and 16% at 5 mM XMP and at 5 mM XMP combined with 1 mM Na_2MO_4, respectively. The effect of 1 mM Na_2MO_4 on XMP nucleotidase was evident: thus based on the UV signal the formation of xanthosine was reduced by 57% (at 5 mM XMP). The other nucleotidase inhibitors tested such as Na_3VO_4, Na_2HAsO_4 and NaF



Scheme 1. Possible routes for the biosynthesis of caffeine. Reactions subject to metabolic channelling are contained in the shaded boxes.

were not as active. Moreover, at 5 mM Na_2MO_4 the formation of [^{14}C]7-methyl-XMP was, as compared with 1 mM Na_2MO_4, not significantly increased because *N*-methyltransferase activity was simultaneously inhibited as demonstrated by the overall reduction of [*Me*-^{14}C] labelled products by 20% (not shown). Similarly, when the assay buffer (0.1 M tricine) was replaced by 0.2 M K_2HPO_4 (not shown), the overall reduction of *N*-methyltransferase activity was 50% and four times less [^{14}C]7-methyl-XMP was formed, although XMP nucleotidase and 7-methyl-XMP nucleotidase were inhibited (as compared with the assay with tricine and no inhibitor) by 95 and 88%, respectively. Finally, we have to record that GMP did not serve as a methyl group acceptor in this modified assay (5 mM GMP, 1 mM Na_2MO_4).

Hence, although the presence of a XMP *N*-methyltransferase in coffee leaflets was clearly proved by this investigation, we cannot report on its net activity, because it was not possible to distinguish between 7-methylxanthosine formed directly (from xanthosine) or produced by hydrolysis of 7-methyl-XMP. The same holds for 7-methylxanthine. To measure the net activity of XMP *N*-methyltransferase, it will be necessary to completely inhibit either XMP nucleotidase (i.e. formation of xanthosine) or 7-methyl-XMP nucleotidase without severely impairing the *N*-methyltransferase reaction. However, it looks as if purification or at least separation from nucleotidases will be a prerequisite to the successful characterization of XMP *N*-methyltransferase.

Caffeine biosynthesis: a new hypothesis

In essence, the *in situ* experiments with leaflets led to the same conclusions as with suspension-cultured cells. Thus, no N-7-monomethylated compound other than 7-methylxanthine was labelled upon feeding the caffeine precursors [^{14}C]adenine and [*Me*-^{14}C]methionine, and therefore, the first methylation has to be metabolically channelled [10]. Since 7-methylxanthine is not formed directly from xanthine ([2, 7, 11] and Table 3), xanthosine and XMP are the most promising candidates for methylation at N-7. Because of its significant methylation by enzyme extracts as shown in the present investigation, XMP is favoured. This view is supported by the fact that purine or pyrimidine base modifications generally take place at the (poly)nucleotide level (*cf* the methylation of dUMP to dTMP by the thymidylate synthase). According to our new hypothesis, IMP formed *de novo* or via the purine salvage pathway, is converted to XMP, which is either hydrolysed to xanthosine and further catabolized to xanthine, uric acid, allantoin, etc. (purine degradation pathway), or enters the caffeine biosynthetic route by methylation to 7-methyl-XMP. However, this latter intermediate is not released (and thus cannot be detected) but immediately metabolized to 7-methylxanthine. Whether this metabolically channelled transformation includes two or three enzyme reactions (XMP → 7-methyl-XMP → 7-methylxanthine or XMP → 7-methyl-XMP → 7-methylxanthosine → 7-methylxanthine, respectively) cannot yet be specified. The postulated reactions are depicted in Scheme 1, in which the metabolic channel sector is shown by shading.

The significance of xanthosine, as indicated by the question mark in the lower part of Scheme 1, is still not fully elucidated. It is certainly an excellent substrate for methylation at N-7 of the purine ring (Table 3). However, as we recently showed by tracer kinetics [10], xanthosine can only be implicated in caffeine biosynthesis if we assume two separate xanthosine pools, i.e. a caffeine precursor pool and a collecting pool. Since this latter xanthosine pool is tremendously expanded in cell cultures treated with adenine [9, 10], it evidently acts as a collecting pool for a surplus of purine moieties and constitutes a part of the purine degradation pathway. In contrast to cell cultures [10], radiolabelled xanthosine as well as the purine catabolites allantoin and uric acid cannot be detected in young coffee leaflets after [^{14}C]adenine feeding indicating that purine catabolism is not significant at this developmental stage. Consequently, the pathways of purine degradation and caffeine biosynthesis are not directly linked by competition for xanthosine, as always supposed [8]. Moreover, we do not think that xanthosine is methylated *in vivo* but rather represents an artificial substrate for *N*-methyltransferase like paraxanthine (1,7-dimethylxanthine), which was proved to be the most active methyl group acceptor among methylxanthines without being a natural precursor of caffeine [7, 11]. *In vivo* methylation of xanthosine is probably prevented by cell compartmentation or metabolic channelling.

EXPERIMENTAL

Chemicals. Radiochemicals were purchased from Amersham (UK) ([U-^{14}C]adenine, 270 mCi mmol^{-1}; *S*-adenosyl-L-[*Me*-^{14}C]methionine, 60 mCi mmol^{-1}) and Du Pont NEN, USA (L-[*Me*-^{14}C]methionine, 45 mCi mmol^{-1}). Nucleotides, 7-methyl-GMP, N-7-methylated nucleosides, purine bases, NAD(H), NADP(H), FAD, SAM, allantoin and uric acid were from Sigma (Mo, USA), and methylxanthines as well as nucleosides from Fluka, Buchs, Switzerland. 7-Methyl-XMP was synthesized as follows.

Chemical synthesis of 7-methylxanthosine 5'-monophosphate (7-methyl-XMP). This was carried out using the procedure slightly modified of Hendler *et al.* [12] for the preparation of 7-Me-GMP: $Na_2XMP.2H_2O$ (150 mg; 0.34 mmol) was dissolved in H_2O (final vol 3 ml) and the pH was adjusted to 4.5 with 1 M HCl. Dimethyl sulphate (204 μl; 2.14 mmol) was added with continuous and vigorous stirring. To minimize esterification of the phosphate group, the pH of the reaction was maintained at 4.0 during the entire period using a pH-stat (Impulsomat E473, Multidosimat E415, Metrohm, Herisau, Switzerland). The course of the reaction was monitored by TLC (silica gel 60 F_{254}, Merck) in satd $(NH_4)_2SO_4$–isopropOH-KPi buffer, pH 7.4 (79:2:19). The R_f values were 0.70 and 0.47 for XMP and 7-methyl-XMP, respectively. After 2 hr, when the reaction reached the equilibrium

between formation of the product and its degradation to methylxanthines, the soln was applied onto an anion exchange column (Dowex 1, X4, 200–400 mesh, 14 × 1.5 cm) previously equilibrated with 0.1 M NH_4–acetate (pH 4.7). Elution (1 ml min^{-1}) was carried out using the same buffer but with a molar strength of 0.4. Methylated xanthines appeared first (10–60 ml, 9% of theory) followed by the methylester of 7-Me-XMP (80–210 ml, 7%), and finally by the desired product 7-Me-XMP (320–610 ml, 57%). The educt (XMP) was not eluted under these conditions. The fractions were combined and the solvent was removed by lyophilization. To convert it into the Na^+-form, the product was dissolved in 1 ml water (*ca* 0.2 M) and the resulting soln was applied onto a column of Dowex 50W-Na^+ (X4, 50–100 mesh, 21 × 0.6 cm). After elution with water (0.1 ml min^{-1}), the ml-frs (2–6) were pooled, concentrated by evap. to 1 ml and brought to pH 8.3 with dil. NaOH. The product 7-Me-XMP which precipitated overnight in the cold was washed with EtOH and Et_2O, and finally dried over P_2O_5. UV λ_{max} (0.1 M phosphate pH 7.0) nm (log ε): 209.5 (4.12), 253.5 (3.92), 286 (3.90); UV λ_{min} (0.1 M phosphate pH 7.0) nm (log ε): 292.5 (3.39), 267 (3.71). Hydrolysis (pH 2, 80°, 30 min) yielded a substance which proved to be identical with 7-methylxanthine (UV spectra and RP-HPLC). 7-Me-XMP was stable at 37° between pH 5 and 7. At pH 4 (fission of the *N*-glycosidic bond) the half life time was 116 hr, and 250 hr at pH 8 (imidazole ring opening).

Plant material. All studies were carried out with the youngest leaflets (*ca* 1–1.5 cm long) from shoot apices of *Coffea arabica* L. var. *catuai* grown in the greenhouse.

Tracer feeding. Whole leaflets (8–12 leaf pairs, *ca* 300 mg fr. wt) were incubated according to [13] in 2 (or 1) ml sterilized buffer containing 30 mM K-Pi buffer (pH 5.6), 10 mM sucrose and 1.5 μCi [U-^{14}C]adenine (or 4 μCi L-[*Me*-^{14}C]methionine) in a 20 ml glass vial while shaking on a gyratory shaker (90 rpm) at 27° in the dark. After 4 and 24 hr, samples of incubation buffer (100 μl) and half of the leaflets (*ca* 150 mg fr. wt) were taken. The medium was filtered through a 0.45 μm filter (Millex-HV 14, Millipore) and directly used for HPLC analysis, whereas leaflets were washed with H_2O and immediately frozen in liquid N_2.

Extraction. Frozen leaflets were homogenized in a mortar under liquid N_2. The resulting powder was extracted in 20 ml 80% MeOH and sonicated for 15 min. After centrifugation (20000 × g for 10 min), the supernatant was evaporated and the residue was dissolved in 500 μl H_2O and, after filtration (Millex-HV 14, 0.45 μm, Millipore), used for HPLC analysis. The pellet containing the nucleic acids was dried under a stream of N_2, hydrolysed in 0.5 ml 1 NHCl for 1 hr at 100° in a sealed test tube and dried again as described above to remove the solvent. Finally, the residue was dissolved in 500 μl H_2O and filtered (see above) prior to HPLC analysis.

PAs are washed out into the incubation buffer and, depending on the incubation time, only 5–40% of the formed [^{14}C]purine alkaloids could be recovered in the leaf extracts. In order to determine the actual incorporation of [^{14}C]adenine into its metabolites it is very important to consider also the washed out [^{14}C]purine alkaloids, see Tables 1 and 2.

Enzymatic studies. Leaflets (*ca* 600 g fr. wt) were homogenized in a chilled mortar with 12 ml 0.1 M tricine–NaOH buffer (pH 7.3) containing 250 mM sucrose, 50 mM NaCl, 2 mM DTT, 2 mM NaEDTA, 0.5% Na ascorbate and 250 mg PVPP (Sigma). The homogenate was centrifuged for 15 min (20000 × g, 4°). From the supernatant (10 ml) the proteins were precipitated by slowly adding solid $(NH_4)_2SO_4$ (70% sat.) at 4°. The ppt. was collected by centrifugation (20000 × g, 4°, 15 min), dissolved in 600 μl buffer and the resulting crude extract was desalted by centrifugation on Sephadex G-25 (Pharmacia) according to ref. [14]. The column (1 × 4 cm, in a plastic syringe barrel), previously equilibrated with 0.05 M tricine–NaOH (pH 7.5) buffer containing 0.05% Na ascorbate, was charged with 300 μl crude extract, put into a tube and centrifuged at 1400 × g for 2 min, this yielding exactly 300 μl eluate (enzyme extract) not diluted by the desalting procedure.

N-methyltransferases were assayed using 50 μl of the desalted enzyme extract, 20 μl 0.5 M tricine–NaOH buffer (pH 7.5), 10 μl 10 mM $MgCl_2$, 10 μl (0.05 μCi) of *S*-adenosyl-L-[*Me*-^{14}C]methionine (60 mCi $mmol^{-1}$) and 10 μl of the 10-fold concentrated substrate (methyl acceptor, 5 and 50 mM, respectively) and nucleotidase inhibitor (10 mM Na_2MO_4) if needed. The incubation was carried out at RT and terminated either after 60 min by the addition 10 μl 1.2 M $HClO_4$ or (to prevent degradation of [^{14}C]7-Me-XMP) by immersing the reaction vial for 45 s into a silicone oil bath at 150°. In the latter case, the reaction was stopped after 20 min and 10 μl 5 mM 7-Me-XMP was added immediately before termination. The protein ppt. was removed by centrifugation. The reaction mixture was analysed by HPLC and on-line radiodetection. This allowed the detection of both the [^{14}C]methylated products of *N*-methyltransferase and the unlabelled metabolites of the substrates, e.g. XMP, formed by nucleotidase or nucleosidase activities in the cell-free extract.

HPLC analysis and radiodetection. This was performed using a liquid chromatograph HP 1090 (Hewlett Packard) equipped with a diode array and connected to a radiodetector (Flo-one, Canberra Packard). Separation was performed on a Nucleosil-100 C18 column (5 μm; 4 × 250 mm; precolumn 4 × 20 mm) with 50 mM ammonium phosphate, pH 3.8, [A] and MeOH–MeCN (1:1) [B] at a total flow rate of 1 ml min^{-1}. The following gradient was used (% B over A): 0–5 min (0); 5–14 min (0–3); 14–25 min (3–30); 25–30 min (30). The column temp. was 40° and the injection vol. 50 μl. Radioactivity was determined on-line in the effluent using 1 ml min^{-1} scintillation liquid. Peaks were identified by comparison of their UV spectra (library established under separating conditions) and R_t values with those of authentic standards. For R_t values of compounds relevant to this study see ref. [10].

SAM (R_t 4.4 min) and 7-Me-XMP (R_t 4.6 min) could not be completely separated by the HPLC system

described above. For an accurate determination of 7-Me-XMP, samples were also analysed using 100 mM ammonium acetate (pH 5.6) instead of 50 mM ammonium phosphate (pH 3.8) as eluent [A]. Under these conditions, the separation of SAM (R_t 7.2 min) and 7-methyl-XMP (R_t 3.6 min) was complete. The identification of 7-Me-XMP was additionally achieved by collecting the corresponding peak after HPLC separation (100 mM ammonium acetate (pH 5.6) was used as eluent [A]) and, after evaporation, hydrolysing it as described above for nucleic acids.

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