Rapid Paper

Methylation of Xanthosine by Tea-leaf Extracts and Caffeine Biosynthesis

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N-Methyltransferase catalyzing the transfer of methyl groups from [$^{14}\text{CH}_3$]SAM to xanthosine, producing 7-methylxanthosine, was extracted in cell-free systems from tea leaves. The purine nucleotides, nucleosides, and bases, except for xanthosine, were all inactive substrates in the methylation at the N-7 position of their purine rings. This confirmed that the methylation of xanthosine to 7-methylxanthosine is the first step of methylation in the pathway for caffeine biosynthesis. The optimum pH for the methyltransferase is 7.5 to 8.0. PCMB (0.5 mM), Zn^{2+} (1 mM) and Cu^{2+} (1 mM) strongly inhibited the enzyme activity. The K_m values for xanthosine and SAM are 0.25 mM at 3.9 μM of SAM and 3.3 μM at 0.40 mM of xanthosine, respectively.

Recent studies^{1~10)} on the biosynthesis of caffeine have shown that the pathway to caffeine is 7-methylxanthosine $\rightarrow$ 7-methylxanthine $\rightarrow$ theobromine $\rightarrow$ caffeine. The step at which the methylation occurs first in the pathway leading to 7-methylxanthosine was unknown. In our previous paper,¹¹⁾ however, results in feeding experiments with tea plants indicated that xanthosine was converted into caffeine *via* 7-methylxanthosine. Subsequently we succeeded in extracting from tea leaves the enzyme involved in the methylation of xanthosine.

In this paper, new evidence for the existence of the enzyme in the extracts is presented, suggesting that the enzyme is involved in the catalysis of the methylation reaction of xanthosine in the pathway for caffeine biosynthesis.

MATERIALS AND METHODS

Materials. The upper three leaves and a bud from fresh tea shoots (*Camellia sinensis*, (L.) O. Kuntze, cv. Yabukita) were plucked at the tea garden near the University of Tsukuba in May, 1983 and stored at -20°C in a freezer. [$^{14}\text{CH}_3$]SAM (59 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. 1-Methylxanthine was prepared by deamination of 1-methylguanine using nitrous acid.¹²⁾ 7-Methyladenine was synthesized from 6-chloropurine by the method of Elion.¹³⁾ Other methylated purine derivatives were all obtained from the Sigma Chemical Co. Alkaline phosphatase (EC 3.1.3.1, from calf intestine) was purchased from Boehringer Mannheim GmbH.

Preparation of cell-free extracts from tea leaves. All the procedures were done at 4°C . Tea leaves (20 g) were homogenized in a blender with 15 g of washed Polyclar AT and 120 ml of 0.1 M PIPES buffer (pH 7.0) containing 0.6% sodium ascorbate. The homogenate was squeezed through two layers of cheesecloth and centrifuged for 20 min at $30,000 \times g$. The supernatant was brought to 80% saturation by addition of solid $(\text{NH}_4)_2\text{SO}_4$ and stirred for 30 min. The precipitate was collected by centrifugation ($30,000 \times g$; 20 min) and dissolved in the PIPES buffer used above. After centrifugation, the supernatant was dialyzed for about 5 hr against 0.01 M PIPES buffer (pH 7.0) containing 0.06% sodium ascorbate. The dialyzed solution was used as the enzyme solution.

Assay for methyltransferase. Enzyme activity was measured using an assay based on the transfer of a ^{14}C -labelled methyl group from [$^{14}\text{CH}_3$]SAM to purine derivatives to produce methylated compounds. The standard assay mixture contained 20 μl of 0.5 M PIPES buffer (pH 7.5), 20 μl of 2 mM xanthosine or other substrates, 10 μl of 10 mM MgCl_2 , 10 μl (about 50,000 dpm) of 39 μM [$^{14}\text{CH}_3$]SAM and enzyme solution and water to 100 μl . The reaction was done at 20°C for 10 min after a preincubation of 5 min and terminated by the addition of 20 μl of 0.6 M HClO_4 .

In the case of XMP, IMP and GMP, the reaction was stopped by heating at 100°C for 30 sec. Part of the reaction mixture was treated with 5 units of alkaline phosphatase (EC 3.1.3.1, from calf intestine) at 37°C for 20 min to analyze the product at the nucleoside level. When AMP and adenosine were used as substrates, the reaction product was hydrolyzed to yield the free base by heating at 100°C in 1 N HCl for 1 hr.

After centrifugation of the mixture at $1,600 \times g$ for 5 min, 20 μl of the supernatant were chromatographed on Whatman No. 1 filter paper in *n*-BuOH-AcOH- H_2O (4:1:1), solvent A. Unlabelled authentic 7-methylxan-

Abbreviation: SAM, S-adenosyl-L-methionine; PCMB, *p*-chloromercuribenzoate; PIPES, Piperazine-*N,N'*-bis(2-ethanesulfonic acid).

thosine and 7-methylxanthine, or other methylated compounds were co-chromatographed as carriers. The spots corresponding to the authentic materials were detected under UV light and cut out from the paper. The segments of chromatograms were allowed to soak in H₂O for 30 min and their radioactivity was measured in a Beckman LS-8100 liquid scintillation counter.

Identification of the reaction product. To determine the site of methylation in the purine ring of the reaction product, the reaction was done at 20°C for 1 hr on a large scale (400 μ l) using 0.5 μ Ci of SAM. The reaction mixture was applied to Whatman No. 1 filter paper in a band and developed in solvent A. The radioactive area was detected using a chromatogram scanner and cut out in a band from the paper. The radioactive material was eluted with water by the descending technique. The first 0.2 ml of the solution, which contained most of the material, were used for the identification. Part of the eluate was hydrolyzed by heating at 100°C in 1 N HCl for 1 hr. Then the isolated reaction product and its hydrolysate were rechromatographed on Whatman No. 1 filter paper in solvent systems as follows: (A) *n*-BuOH-AcOH-H₂O (4:1:1), (B) *n*-BuOH-isobutyric acid-H₂O-28% NH₄OH (30:15:10:1), (C) *tert*-BuOH-methyl ethyl ketone-H₂O-28% NH₄OH (4:3:2:1) and (D) 0.1 M borate buffer (pH 9). The spots of the authentic compounds and radioactive products were detected by UV light and a chromatogram scanner, respectively.

RESULTS AND DISCUSSION

Formation of 7-methylxanthosine from xanthosine

Suzuki and Takahashi³⁾ have demonstrated the existence of *N*-methyltransferases which produce theobromine from 7-methylxanthine and caffeine from theobromine in cell-free extracts from tea leaves, but they could not detect the activity of the enzyme involved in the methylation of xanthosine. Similar results have been reported by Roberts and Waller⁷⁾ using cell-free extracts from coffee fruits. However, by applying the method described above, we were able to extract the enzyme catalyzing the methylation of xanthosine.

To identify the reaction product of methylation by *N*-methyltransferase, paper chromatographic analysis of the product and its hydrolysate was done and it was demonstrated that xanthosine was methylated at the N-7 position (Table I).

The cell-free extracts reproduced all the

TABLE I. IDENTIFICATION OF METHYLATED PRODUCT OF XANTHOSINE BY PAPER CHROMATOGRAPHY

Compound	R _f value			
	Solvent system			
	A	B	C	D
Product	0.18	0.17		
7-Methylxanthosine	0.18	0.16		
7-Methylinosine	0.20	0.34		
7-Methylguanosine	0.25	0.40		
Hydrolysate of product	0.41		0.30	0.60
7-Methylxanthine	0.41		0.31	0.60
3-Methylxanthine	0.43		0.53	0.51
1-Methylxanthine	0.53		0.46	0.58

FIG. 1. Conversion of Xanthosine into Caffeine by Cell-free Extracts from Tea Leaves.

The reaction mixture contained 50 μ l each of 0.5 M PIPES buffer (pH 7.5), 2 mM xanthosine and 10 mM MgCl₂, 100 μ l (0.23 μ Ci) of 39 μ M SAM, and 250 μ l of enzyme solution. The reaction was done at 30°C. Forty microliters of the reaction mixture were pipetted out at each period of incubation and the reaction was terminated by the addition of 20 μ l of 0.3 M HClO₄. Analytical method was that described in the text. The values of 7-methylxanthosine were not corrected for the amount reduced by the inactivation of *N*-methyltransferase. ○, 7-methylxanthosine; ●, 7-methylxanthine; △, theobromine; ▲, caffeine.

reactions in the pathway from xanthosine to caffeine, as shown in Fig. 1, indicating that they contain the complete enzyme system involved in the biosynthesis of caffeine, that is, three *N*-methyltransferase activities and a

FIG. 2. Effect of Temperature on the Enzyme Activity. The standard assay mixture was incubated for 10 min at various temperatures.

FIG. 3. Effect of pH on the Enzyme Activity.

The reaction was done under the standard assay conditions. The buffers used were sodium acetate (○), PIPES-NaOH (●), Tricine*-NaOH (□) and Tris-HCl (■).

* Tricine: Tris(hydroxymethyl)methylglycine.

nucleosidase or nucleoside phosphorylase activity.

Properties of N-methyltransferase catalyzing the reaction from xanthosine to 7-methylxanthosine

The enzyme extracts after dialysis lost about 50, 80 and 100% of their activity over a period of 12, 24, and 72 hr, respectively, at 4°C, whereas the storage at -20°C enabled us to preserve more than 80% of the activity over

TABLE II. METHYLATION OF PURINE COMPOUNDS BY CELL-FREE EXTRACTS FROM TEA LEAVES

The reaction was done under the standard assay conditions described in the text. The final concentrations of four non-methylated bases were 0.5 mM and the others were 1 mM.

Substrate	Methylated product ^a	Relative methylation (%)
XMP	7-Methylxanthosine	(12 ^b)
IMP	—	0
GMP	—	0
AMP	—	0
Xanthosine	7-Methylxanthosine	100
Inosine	—	0
Guanosine	—	0
Adenosine	—	0
Xanthine	3-Methylxanthine	29
Hypoxanthine	—	0
Guanine	—	0
Adenine	—	0
7-Methylxanthine	Theobromine	180
Theobromine	Caffeine	36

^a When compounds other than xanthine derivatives were used as substrates, only 7-methylated products were estimated.

^b 7-Methylxanthosine was detected as the methylated product, but its amount did not increase after alkaline phosphatase (EC 3.1.3.1, from calf intestine) treatment. This fact suggests that XMP is not methylated directly. Therefore, this value corresponds to the rate of methylation of xanthosine produced from XMP by the nucleotidase contained in the cell-free extracts.

72 hr. Analysis of the effect of temperature on the enzyme activity showed that the maximum activity occurred at 20°C and the activity rapidly decreased above 30°C (Fig. 2). The optimum pH for enzyme activity was 7.5 to 8.0 (Fig. 3), which is slightly lower than the pH for the enzymes catalyzing other methylation steps.³⁾ Significant inhibition by thiol reagents was observed, PCMB (100% inhibition at 0.5 mM) being the most effective. At a concentration of 1 mM, Zn²⁺ and Cu²⁺ also completely inhibited the enzyme activity. Mg²⁺ and EDTA had no effect.

The rate of production of 7-methylxanthosine at various concentrations of xanthosine and SAM was deduced from the Lineweaver-Burk plot. The *K_m* values for

xanthosine and SAM were 0.25 mM at 3.9 μ M of SAM and 3.3 μ M at 0.4 mM of xanthosine, respectively, although these values tended to vary with the concentrations of other substrates.

Methylation of purine compounds and the pathway for caffeine biosynthesis

To investigate the first step of successive methylations in the pathway for caffeine biosynthesis, purine nucleotides, nucleosides, and bases were all tested for the presence of methyl acceptors at their N-7 position.

Table II shows that only xanthosine was an active substrate for enzymatic methylation at the N-7 position of the purine ring. In the case of XMP, 7-methylxanthosine was detected as the methylated products, but no increase in the amount of 7-methylxanthosine was observed after treatment with alkaline phosphatase (EC 3.1.3.1, from calf intestine). This suggests that XMP was not methylated directly. The formation of 7-methylxanthosine from XMP was associated with methylation of xanthosine produced from XMP by the nucleotidase contained in the cell-free extracts, which degraded 8% of XMP under the standard assay conditions.

Xanthine was methylated at the N-3 position, but not at the N-7 position (Table II). However, it had already been demonstrated that 3-methylxanthine was an inactive substrate for methyltransferase in the synthesis of caffeine.³⁾ It had also been shown by *in vivo* experiments²⁾ that caffeine was not derived directly from xanthine. In our previous paper,¹¹⁾ we demonstrated the conversion of

xanthosine into caffeine *via* 7-methylxanthosine, using excised tea shoots. Consequently, it was inferred that the substance which is methylated at the N-7 position of the purine ring both *in vivo* and *in vitro* is xanthosine. Furthermore, as shown in Table II, free purine nucleotides were not methylated although other authors^{1,5,6)} suggested that the methylation occurs at the nucleotide level. These results confirm that the methylation occurs first at the step from xanthosine to 7-methylxanthosine in the pathway for the biosynthesis of caffeine.

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