Rapid Paper

Conversion of Xanthosine into Caffeine in Tea Plants

Osamu NEGISHI, Tetsuo OZAWA
and Hiroshi IMAGAWA*Institute of Applied Biochemistry,
The University of Tsukuba,
Sakura-mura, Niihari-gun,
Ibaraki 305, Japan*

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¹⁴C-labelled methionine, xanthosine, and 7-methylxanthosine were given to excised tea shoots. The methyl group of methionine was incorporated into 7-methylxanthosine (ca. 10%) in the earlier period of incubation after the uptake. About 50% of the radioactivity of xanthosine was rapidly incorporated into caffeine via 7-methylxanthosine, 7-methylxanthine, and theobromine within 24 hr. 7-Methylxanthosine was also converted into caffeine at a high rate. The results suggest that the pathway for caffeine biosynthesis is as follows: xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine.

It has been shown that caffeine (1,3,7-trimethylxanthine) is synthesized from 7-methylxanthine via theobromine (3,7-dimethylxanthine) in the tea plant^{1,2)} and the coffee plant.^{3,4)} Furthermore, the purine ring in caffeine is derived from the purine nucleotides in the nucleotide pool rather than in nucleic acids^{2,5)} and xanthine cannot serve as the direct precursor of 7-methylxanthine.⁶⁾ Baumann *et al.*⁷⁾ indicated that 7-methylxanthosine was a precursor of caffeine in the coffee plant and this finding was confirmed by the experiments with cell-free extracts from coffee fruits³⁾ and callus cultures.⁸⁾ However, the pathway leading to the formation of 7-methylxanthosine from the purine nucleotides still remains unclear.

On the other hand, Looser *et al.*⁴⁾ indicated that xanthosine, 7-methylinosine, and 7-methylguanosine could not serve as the precursors of caffeine in the coffee plant. Suzuki and Takahashi¹⁾ obtained negative results for the methylation of xanthosine and XMP using cell-free extracts from tea leaves. Based on

these results it is generally considered that methylation for caffeine formation occurs first in the purine nucleotides.^{4,5,7)}

We were able to obtain results differing from previous findings by feeding a tea plant with ¹⁴C-labelled 7-methylxanthosine, methionine, and xanthosine. In this report, the conversion of these compounds into caffeine and the pathway for caffeine biosynthesis are described.

MATERIALS AND METHODS

Chemicals. L-[methyl-¹⁴C]Methionine (60.4 mCi/mmol) and [2-¹⁴C]xanthosine (49.2 mCi/mmol) were purchased from the Radiochemical Centre, Amersham and Commissariat à l'Énergie Atomique, Paris, respectively. 7-Methyl-[2-¹⁴C]xanthosine was prepared from [2-¹⁴C]xanthosine with a small modification of the technique described by Baumann *et al.*⁷⁾ For the separation of 7-methylxanthosine from xanthosine and 7-methylxanthine the reaction mixture was passed through a column (0.5 × 5 cm) of Dowex 1-X8 (HCOO⁻ form). The fraction containing 7-methylxanthosine was then lyophilized. Labelled 7-methylxanthosine gave a 69.9% yield and the radiochemical purity determined by paper chromatography with *sec*-BuOH saturated with H₂O was 93%. The specific activity was 0.96 mCi/mmol.

7-Methylxanthosine, 1-methylguanosine, and 3-methylxanthine was obtained from the Sigma Chemical Co. 1-Methylxanthine was prepared by the deamination of 1-methylguanine with nitrous acid.⁹⁾

Feeding experiment. Fresh shoots from tea plants (*Camellia sinensis*, (L.) O. Kuntze, cv. Yabukita) cultivated in a tea garden were used. The cut end of each excised shoot (ca. 1 g), consisting of three leaves and a bud, was placed in a small conical vial containing 1 μCi/0.2 ml of 7-methyl-[2-¹⁴C]xanthosine, 2 μCi/0.2 ml of L-[methyl-¹⁴C]methionine, or 1 μCi/0.2 ml of [2-¹⁴C]xanthosine. The experiments were carried out under an illumination of 16,000 lux in a growth cabinet. After absorption of the radioactive compound for ca. 40 min at 35°C and ca. 40% humidity, the shoots were transferred to H₂O in small Erlenmeyer flasks and incubated for various periods of time at 25°C and ca. 60% humidity. At the end of the incubation periods two shoots were removed and frozen until required.

Extraction and analysis of acid-soluble materials. Two shoot tips (ca. 2 g) were homogenized in a chilled mortar with 8 ml of cold 0.2 M HClO₄. After centrifugation at 2,200 × g for 5 min the supernatant was adjusted to pH 4~5 with solid KHCO₃. After further centrifugation

(2,200 × g; 10 min), 20 µl of the supernatant were applied to Whatman No. 1 filter paper and two-dimensional paper chromatography was done with the following solvent systems: (1) *n*-BuOH–AcOH–H₂O (4:1:1) in the first direction and (2) *n*-BuOH–isobutyric acid–H₂O–28% NH₄OH (30:15:10:1), (3) isobutyric acid–0.5 N NH₄OH (5:3), or 2% AcOH in the second direction. Unlabelled authentic materials were co-chromatographed on each sheet as carriers. Radioactive areas on the paper chromatograms were detected by autoradiography. The spots corresponding to the authentic materials, which were detected under UV light, were 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. The activity of each of the ¹⁴C-labelled products in the HClO₄ extract was calculated by assuming that the water content of tea leaf is 80% and the recovery rate of the labelled compound from the homogenate is 90%. The recovery rate was determined by the addition of an adequate amount of [8-¹⁴C]adenine to the homogenate.

Identification of 7-methylxanthosine produced by feeding with L-[methyl-¹⁴C]methionine. To identify 7-methylxanthosine which was detected on the autoradiogram of the extracts from the tea shoots fed with L-[methyl-¹⁴C]methionine, it was isolated from the extracts. After removal polyphenolic compounds from the solution containing acid-soluble materials by extraction with *n*-BuOH, the aqueous solution was fractionated by chromatography

on a column of Dowex 50W-X8 (H⁺ form). Elution was done stepwise with 0.01, 0.05, 0.1, 0.2, and 0.4 M triethylammonium formate buffer (pH 3.8). The radioactive fraction corresponding to 7-methylxanthosine, which was eluted with 0.1 M buffer, was treated with 1 N HCl at 100°C for 1 hr and identified by co-chromatography with authentic methylated xanthines in solvent (1) and *tert*-BuOH–methyl ethyl ketone–H₂O–28% NH₄OH (4:3:2:1), solvent (5).

RESULTS AND DISCUSSION

The results of the feeding experiments are summarized in Fig. 1. The radioactivity of 7-methyl-[2-¹⁴C]xanthosine, L-[methyl-¹⁴C]methionine, and [2-¹⁴C]xanthosine was incorporated into caffeine.

7-Methylxanthosine was converted into caffeine *via* 7-methylxanthine and theobromine (Fig. 1a), indicating that 7-methylxanthosine is a precursor of caffeine in the tea plant as well as in the coffee plant. Incorporation of 7-methylxanthosine into caffeine in the tea plant (*ca.* 55% after 24 hr) was much higher than that in the coffee plant (26% after 8 days).⁷⁾

When tea shoot tips were fed with labelled methionine, a considerable amount of radioac-

FIG. 1. Distribution of Radioactivity among Xanthosine (▲), 7-Methylxanthosine (■), 7-Methylxanthine (□), Theobromine (●), and Caffeine (○) When Groups of Two Excised Tea Shoots (*ca.* 2 g) Were Each Fed with 2 µCi of 7-Methyl-[2-¹⁴C]xanthosine (0.96 mCi/mmol) (a), 4 µCi of L-[methyl-¹⁴C]Methionine (60.4 mCi/mmol) (b), or 2 µCi of [2-¹⁴C]Xanthosine (49.2 mCi/mmol) (c) for *ca.* 40 min, Followed by Incubation in H₂O for Various Periods of Time.

tivity (ca. 10% of the ^{14}C supplied) was found in 7-methylxanthosine at the earlier period of incubation after the uptake (Fig. 1b). Almost all of the labelled 7-methylxanthosine was converted into caffeine after incubation. No spots of methylated nucleotides could be detected on the autoradiogram in the area where they were expected to occur.

The 7-methylxanthosine found in this experiment was also identified after hydrolyzing to 7-methylxanthine, since the definite result for determination of the position of methyl group at the xanthine ring can be obtained by this method. The hydrolysis product matched 7-methylxanthine (R_f 0.40 in solvent (1) and 0.30 in solvent (5)).

These results suggest that 7-methylxanthosine is the first compound to be methylated in the biosynthesis of caffeine, and that xanthosine is a direct precursor of 7-methylxanthosine, although xanthosine has been considered to be an inactive substrate for methylation *via* methyltransferase^{1,3)} or an inactive precursor of caffeine.⁴⁾

Figure 1c clearly demonstrated that xanthosine is also an intermediate compound in the biosynthesis of caffeine. The radioactivity of xanthosine was incorporated into caffeine at the high rate of about 50% within 24 hr. Xanthosine disappeared within 1 hr after the uptake, but the amount of theobromine rapidly increased. 7-Methylxanthosine and 7-methylxanthine also appeared in small amounts at the earlier period of incubation. This supports the view that 7-methylxanthosine is formed from xanthosine. In the feeding experiment with 7-methylxanthosine, the conversion of 7-methylxanthosine to theobromine appeared to proceed slowly, compared with that of xanthosine in this experiment. This phenomenon may be ascribed to the fact that the specific activity of 7-methylxanthosine (0.96 mCi/mmol) synthesized chemically is so low, one fiftieth of that of xanthosine (49.2 mCi/mmol), that the conversion of large amounts of 7-methylxanthosine

would require much time to take place.

Our results suggest that the pathway for caffeine biosynthesis is as follows: xanthosine $\rightarrow$ 7-methylxanthosine $\rightarrow$ 7-methylxanthine $\rightarrow$ theobromine $\rightarrow$ caffeine. Although it is also possible that caffeine is derived from the methylated nucleotides in the nucleotide pool,^{4,5,7)} it can be inferred that methylation occurs first at the step of the reaction from xanthosine to 7-methylxanthosine. Presently studies are being done in our laboratory to determine whether *N*-methyltransferase plays a role in this reaction in tea leaf extracts.

On the other hand, it has been shown in the tea plant that the radioactivity of [$8\text{-}^{14}\text{C}$]-adenine, [$8\text{-}^{14}\text{C}$]-guanine, and [$8\text{-}^{14}\text{C}$]-hypoxanthine is incorporated into caffeine *via* purine nucleotides.^{2,6)} The following explanation can account for such findings in relation to the process of methylation. Each base may be converted into the corresponding nucleotide *via* the pathway of purine salvage, and interreaction between purine nucleotides could occur, resulting in the formation of xanthosine *via* XMP. Subsequently the methylation of xanthosine would lead to the biosynthesis of caffeine.

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