7-METHYLXANTHOSINE—AN INTERMEDIATE IN CAFFEINE BIOSYNTHESIS

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Absract—Ring-labelled 7-methylxanthosine was synthesized and fed to leaves of *Coffea arabica*. Up to 26% of the tracer was converted into caffeine within 8 days.

INTRODUCTION

Based on results from leaf disk experiments in which potential, non-radioactive precursors together with L-methionine-[Me-^{14}C] were fed, we proposed a pathway for caffeine biosynthesis [1]. According to this, the nucleoside 7-methylxanthosine is converted by hydrolysis of the *N*-glycosidic bond to the corresponding base 7-methylxanthine which is methylated via theobromine (3,7-dimethylxanthine) to caffeine (1,3,7-trimethylxanthine). The two final methylation steps have been demonstrated with cell-free extracts of the tea plant [2].

The aim of the present communication is to examine the precursor nature assigned to 7-methylxanthosine in caffeine biosynthesis. For this reason we synthesized the radioactive form of this compound and studied the ^{14}C-incorporation into caffeine after feeding leaves attached to the coffee plant.

RESULTS AND DISCUSSION

The results are summarized in Table 1. Dry wt and caffeine content increased slightly during the experiment. Since for technical reasons, the amount of label injected varies from leaf to leaf, total as well as specific radioactivities found in caffeine cannot be compared directly. The percentage of ^{14}C-activity found in caffeine related to the applied radioactivity was 8, 14, 23 and 26% after 1, 2, 4 and 8 days feeding respectively. In a preliminary experiment with an almost 20 times lower sp. act. of 7-methylxanthosine (0.15 mCi/mmol), relative incorporation was 25% after 7 days but the kinetics were still linear [3]. Hence, we suppose that some of the tracer is catabolized on the way to the site of caffeine biosynthesis. The values for theobromine were not plotted as the extraction method used recovers only *ca* 5% of it.

The results confirm 7-methylxanthosine to be an intermediate in caffeine biosynthesis as was also earlier proposed by Ogutuga and Northcote [4]. The question now arises from which precursor this nucleoside is formed. Since in our earlier test system, feeding of 7-methylinosine did not increase the ^{14}C-incorporation into caffeine [1], one could regard 7-methyl XMP as precursor. Its formation from XMP is unlikely, because this nucleotide was shown to be inactive in *in vitro* enzymatic methylation studies by Suzuki and Takahashi [2]. However, it is doubtful whether such results from the tea plant are also valid for the coffee plant. Moreover, negative findings with enzyme preparations are not conclusive for the actual *in vivo* situation. The same authors have studied the purine bases hypoxanthine, xanthine, guanine and adenine for their precursor nature in caffeine biosynthesis of the tea plant [5, 6]. Among them adenine-[^{14}C] feeding was the most effective, resulting in relatively high incorporation of radioactivity into caffeine and its direct precursors, theobromine and 7-methylxanthine. This observation drew our attention to a scarcely cited hypothesis made by Kremers in 1954 [7]. According to this, trigonelline and caffeine would have their common origin in one and the same molecule, namely in the coenzyme NAD. In the case of caffeine biosynthesis he proposes that adenine is hydrolytically released, deaminated and further oxidized to xanthine which in turn is methylated to caffeine. Today we know that xanthine cannot be methylated to caffeine by the tea or the coffee plant.

Table 1. Analysis of leaves fed with 7-methylxanthosine-[2-^{14}C]

Time (days)	Dry wt (mg)	7-Methylxanthosine-[2-^{14}C] injected (dpm × 10^6)	Caffeine isolated (μg)	Radioactivity in caffeine total (dpm)	Radioactivity in caffeine specific (dpm/μg)
1	520	1.04	7560	90 000	11.9
2	570	1.18	7980	174 400	21.9
4	570	0.82	7770	187 500	24.1
8	590	1.08	8400	284 000	33.8

If one still adheres to the basic concept of this hypothesis, which has never been proved or disproved, one could consider two possible pathways. (1) Methylation of the adenine residue in the coenzyme at N-7; release of 7-methylAMP and conversion to 7-methylXMP via 7-methylIMP. (2) Deamination of the adenine residue leading to nicotineamide-hypoxanthine-dinucleotide (NHD); methylation of the hypoxanthine residue or, if oxidized, of the xanthine residue at N-7; release of the corresponding nucleotide 7-methylIMP or 7-methylXMP. From the chemical point of view the latter methylations are preferable to N-7 methylation of the adenine residue [8]. Finally we must add that not only all the coenzymes but also the polynucleotides containing adenine should be included in the working hypothesis.

EXPERIMENTAL

Synthesis of 7-methylxanthosine-[2-^{14}C]. The procedure of ref. [8] was modified to a micro scale prepn Xanthosine-[2-^{14}C] (49.2 mCi/mmol, Commissariat de l'Energie Atomique, Paris, France) was diluted with 'cold' xanthosine to a sp. act. of 2.84 mCi/mmol in order to minimize self-decomposition of the end product. 10 μCi (1 mg) were pipetted into a pointed vial (4 × 30 mm) and the solvent (H_2O) was evapd under red. pres. After addition of 10 μl *N,N*-dimethylacetamide and 1 μl $(Me)_2SO_4$ the reaction mixture was shaken vigorously for 90 min. H_2O (40 μl) was added and TLC was carried out (Si gel F 254; MeOH *iso*PrOH–H_2O, 8:1:1) with 7-methylxanthosine (Sigma, St. Louis, USA), 7-methylxanthine (Fluka, Buchs, Switzerland) and xanthosine as reference substances. Radioactivity distribution and relative yield was determined by radiochromatogram scanning: 7-methylxanthosine (R_f 0.38) 69%, 7-methylxanthine (R_f 0.53) 12%, xanthosine (R_f 0.60) 7% and unknown compounds (R_f < 0.22) 12%. The aq. eluate (40 ml) from the band corresponding to 7-methylxanthosine was filtered (cellulose nitrate 0.1 μm) and the resulting filtrate was lyophilized. To determine radiochemical purity a part of the product was chromatographed in a second solvent (TL Si gel, H_2O satd 2-BuOH). Based on peak integration a value of 93% was obtained.

Feeding and extraction techniques. Coffee plants (*Coffea arabica* cv caturra) were cultivated in 13 hr light, 140 W/m^2, 24°, 55% rel. humidity; 11 hr darkness, 18.5°, 75% rel. humidity. Each of the second youngest leaf pairs on two opposite branches of a 21-month-old plant was fed with 7-methylxanthosine-[2-^{14}C] (2.84 mCi/mmol, 2.5 × 10^6 dpm/ml) by the use of Hagborg's device [9] which allows injection through the stomata with almost no loss. At intervals of 1, 2, 4 and 8 days after feeding, one of the leaves was harvested and dried (10 hr, 90°). To examine chemical stability of the tracer during the time of the expt a part of the feeding soln was buffered (pH 6.5, 1/15 M Na-K-Pi), sterile filtered (cellulose nitrate 0.2 μm) and exposed to the same conditions as those used for the plants. TLC of aliquots (Si gel, H_2O satd 2-BuOH) proved stability during the expt.

Caffeine was isolating according to [10], modified. The finely ground leaf powder was refluxed for 20 min with 12.5 ml 0.01 N H_2SO_4, mixed with 1 g MgO, cooled and filtered through glass filter G 4. Caffeine was removed by shaking the filtrate 3 × with 0.5 vol. of $CHCl_3$. Following evapn of $CHCl_3$ the residue was dissolved in $CHCl_3$ and chromatographed (TL Si gel; $CHCl_3$–MeOH, 9:1). Radioactivity distribution was monitored with a radiochromatogram scanner. Caffeine (R_f 0.49) was eluted (H_2O) and quantitated spectrophotometrically at 271 nm. Radioactivity was measured by liquid scintillation counting in Bray's soln [11] and dpm values were calculated by the channel ratio.

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