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THE BIOSYNTHESIS OF CAFFEINE IN THE COFFEE PLANT

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Key Word Index—*Coffea arabica*; Rubiaceae; coffee; biosynthesis; methylated xanthines; caffeine.

Abstract—Leaf disks of *Coffea arabica* were infiltrated simultaneously with L-methionine-(methyl-^{14}C) and with various possible precursors of caffeine biosynthesis. The results permit the identification of theobromine, 7-methylxanthine and 7-methylxanthosine as precursors of caffeine. 7-methylguanosine seems not to be an intermediate in caffeine formation.

INTRODUCTION

PURINE ring biosynthesis of caffeine (1,3,7-trimethylxanthine) has been investigated by Anderson and Gibbs[1] in coffee leaves and by Preusser and Serenkov[2] in tea leaves. These were incorporation studies using labelled precursors and indicated that ring formation of caffeine follows the classical scheme of purine nucleotide biosynthesis.[3] However, the question whether caffeine is formed directly from the purine pool or as a degradation product of nucleic acids—in the first place RNA—was not touched upon in these experiments. In 1970 Ogutuga and Northcote[4] proposed two biosynthetic pathways for caffeine: "Pathway I" from the purine pool via xanthine, 3-methylxanthine and 1,3-dimethylxanthine to caffeine, and "Pathway II" from the purine pool via nucleic acids (methylation of the guanine residue at N7), 7-methylguanylic acid, 7-methylguanosine, 7-methylxanthosine, 7-methylxanthine and 3,7-dimethylxanthine (theobromine) to caffeine. Based upon isotope incorporation kinetics after continuous and pulse feeding of tea callus tissue with $^{14}CO_2$ and L-methionine-(methyl-^{14}C), the authors consider "Pathway II" the more likely. Even though caffeine can be metabolized by the coffee plant,[5,6] this purine alkaloid may still be regarded—at least in short term experiments—as the end product of a long biosynthetic pathway. In the present work we have assumed that, by addition of a compound which can be utilized by the tissue for caffeine biosynthesis, the direction of the reactions would be shifted in favour of the end product. To monitor caffeine synthesis we have chosen methylation with radioactive methyl groups. For this purpose leaf disks were infiltrated with presumed precursors and simultaneously with L-methionine-(methyl-^{14}C). In this manner we were able to determine the individual intermediates of a likely pathway of caffeine synthesis as far back as the nucleoside 7-methylxanthosine.

[1] ANDERSON, L. and GIBBS, M. (1962) *J. Biol. Chem.* **237**, 1941.
[2] PREUSSER, E. and SERENKOV, G. P. (1963) *Biokhimiya* **28**, 857.
[3] BUCHANAN, J. M. and HARTMAN, S. C. (1959) *Adv. Enzymol.* **21**, 199.
[4] OGUTUGA, D. B. A. and NORTHCOTE, D. H. (1970) *Biochem. J.* **117**, 715.
[5] KALBERER, P. (1965) *Nature* **205**, 597.
[6] BAUMANN, T. W. and WANNER, H. (1972) *Planta* **108**, 11.

RESULTS AND DISCUSSION

The experiments were carried out during a 2-yr period. The varying caffeine contents from one experiment to the other are due to seasonal variations, with especially low values being encountered in the winter, largely caused by temperature fluctuations in the greenhouse.[7] In our case the temperature extremes were 18 and 30°.

Time course of ^{14}C-incorporation

Leaf disks were fed with 20 μCi/ml L-methionine-(methyl-^{14}C). The average uptake by each group of six disks was $8{\cdot}9 \times 10^6$ dpm. The ^{14}C-incorporation into caffeine increases almost linearly with time and reaches about 1% of the uptake after 32 hr (Fig. 1). It has been repeatedly shown that L-methionine can act as a methyl group donator in caffeine biosynthesis.[1,4,8] We were unable to detect methionine by the ninhydrin reaction in leaf disk extracts after paper chromatography. The administered radioactive methionine is already metabolized within two hours after infiltration. However, an unknown compound appears (R_f 0·28; *n*-BuOH–HOAc–H_2O, 4:1:1) in which a high percentage of the ^{14}C-activity taken up is found during the entire experiment. A similar observation in the tea plant was described recently.[9]

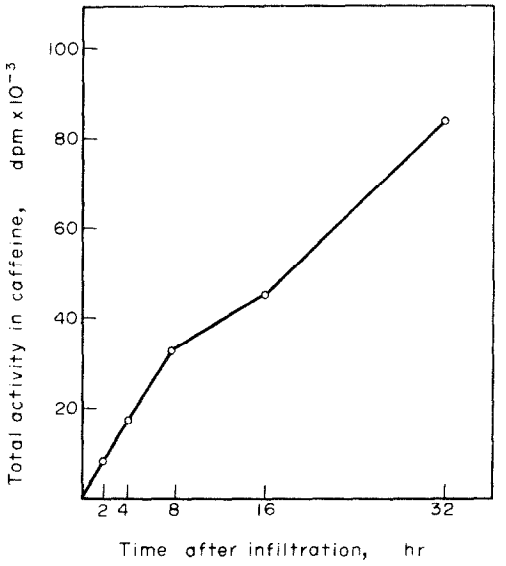

FIG. 1. INCORPORATION OF ^{14}C FROM L-METHIONINE-(METHYL-^{14}C) INTO CAFFEINE.

Experiments with potential precursors

The leaf disks were analyzed after illumination for 12 hr. The two dimethylxanthines theophylline (1,3-substituted) and theobromine (3,7-substituted) are generally considered to be direct precursors of caffeine. They cannot be found in the leaves by routine examination, although their existence in this genus in extremely small quantities has been proved by analysis of large amounts of crude coffee beans.[10] The leaf disks were infiltrated with

[7] PREUSSER, E. (1967) *Biol. Zentralblatt* **86**, 485.
[8] INOUE, T. and ADACHI, F. (1962) *Chem. Pharm. Bull.* **10**, 1212.
[9] SUZUKI, T. (1973) *Biochem. J.* **132**, 753.
[10] FRANZKE, C., GRUNERT, S., HILDEBRANDT, U. and GRIEHL, H. (1967) *Hoppe-Seiler's Z. Physiol. Chem.* **348**, 1725.

2·8 mM solutions of these two compounds containing 7 μCi/ml L-methionine-(methyl-^{14}C). Water with the tracer alone served as control. Compared with the control, the addition of theobromine enhances the ^{14}C-incorporation into caffeine about 20-fold (Table 1, Series I). Theophylline has no effect. From our analysis (unpublished) of wild coffee species we know that caffeine is replaced by theobromine in the leaves of *C. lancifolia* subsp. *auriculata* Chevalier. Obviously this species is not able to perform the last methylation step leading to caffeine. That theobromine is the direct precursor of caffeine, is also supported by the recent work of Suzuki.[9]

TABLE 1. TESTING OF POSSIBLE PRECURSORS

Substance tested	^{14}C-Methionine uptake (dpm × 10^{-6})	Caffeine isolated (μg)	Total activity in caffeine (dpm)	Sp. act. of caffeine (dpm/μg)
		Series 1		
Control (H_2O)	3·10	570	32000	56
Theophylline	2·15	760	30000	40
Theobromine	3·75	670	680000	1015
		Series 2		
Control (H_2O)	2·45	1020	32000	31
Theobromine	2·60	810	254000	314
Xanthine	3·05	960	26000	27
1-Methylxanthine	2·10	890	29000	33
7-Methylxanthine	2·40	1040	176000	169
Xanthosine	3·00	830	36000	43
7-Methylxanthosine	2·90	1110	124000	112
Guanosine	2·55	960	37000	39
		Series 3		
Control (H_2O)	4·55	210	21600	103
7-Methylxanthosine	4·50	240	554000	2308
7-Methylguanosine	4·10	240	26000	108
7-Methylinosine	4·20	180	12100	67

In the next experiment (Table 1, Series 2) we tested a larger number of compounds. As a consequence of the low water solubility of xanthine we were forced to use 0·5 mM solutions. Tracer supply was the same as above. Although 3-methylxanthine was not available for testing, existing results make it certain that the methyl group in position 7 is first introduced, as already postulated by Anderson and Gibbs.[1] This assumption is supported by the observation that 7-methylxanthine as well as theobromine accumulate in the culture medium of growing tea callus tissue.[4] Thus the synthesis of radioactive caffeine is increasingly stimulated by the compounds 7-methylxanthosine, 7-methylxanthine and 3,7-dimethylxanthine, in this order. These findings support "Pathway II" of caffeine biosynthesis proposed by Ogutuga and Northcote,[4] in which methylation should occur at N7 of guanine in a polynucleotide, and in which subsequently 7-methylguanylic acid is transformed via 7-methylguanosine to 7-methylxanthosine. It was therefore necessary to test 7-methylguanosine in our system. Table 1, Series 3 shows the result of this experiment, in which methylated nucleosides were given as 13·5 mM solutions. The ^{14}C-methionine activity was 10 μCi/ml. 7-methylguanosine as well as 7-methylinosine have no stimulatory effect. We should also mention that feeding of 7-methylxanthine and 7-methylxanthosine leads, as expected, to formation of highly radioactive theobromine, 20'200 dpm being found in the last experiment after 7-methylxanthosine feeding.

On the basis of these results, we are convinced that 7-methylguanosine is not an intermediate in caffeine biosynthesis. However this in no way disproves the above mentioned hypothesis postulating nucleic acids as precursors. It is possible that the phosphate group is cleaved from the nucleotide only after conversion of 7-methylguanylic acid to 7-methylxanthylic acid. As an alternative we consider methylation of IMP (from the purine nucleotide biosynthesis) or of its derivatives XMP and GMP. Studies with nucleotides may solve this problem.

EXPERIMENTAL

Precursor feeding. Disks, 1·8 cm dia, were cut out from fully developed leaves of 2- and 3-yr-old plants, thoroughly mixed, and vacuum infiltrated with the test soln. Together with L-methionine-(methyl-^{14}C), each of the following substances was administered: guanine, 7-methylguanine, xanthine, 3,7-dimethylxanthine, 7-methylguanosine, 7-methylxanthosine, 1-methylxanthine, xanthosine, 1,3-dimethylxanthine, guanosine, 7-methylxanthine, 7-methylinosine. When infiltration was complete (2 min), the leaf disks were blotted with filter paper. To estimate isotope uptake, the radioactivity of filter paper and of residual solution was subtracted from the initial supply. After evaporation of intercellular water (30 min) the leaf disks were transferred, upside down, into a Petri dish with the lid lined with wet filter paper, and illuminated from below with a 40 W fluorescent lamp at a distance of 40 cm.

Extraction procedures. Caffeine, and in some cases also amino acids, was isolated by the following procedure. Leaf disks were frozen in liquid N_2, ground in a mortar, extracted for 1 hr with 5 ml 70% EtOH at 4° and rinsed with 5 ml 70% EtOH. The EtOH extracts were combined and extracted with $CHCl_3$ (× 2). The $CHCl_3$ phases were combined and extracted with 15 ml H_2O. The aqueous phase was recombined with the alcoholic phase and the amino acids isolated according to Virtanen.[11] The $CHCl_3$ phase was evaporated and caffeine was isolated (after Kogan *et al.*,[12] slightly modified) from the residue, which was boiled 20 min with 12·5 ml 0·01 N H_2SO_4, mixed with 1,3 g MgO, cooled and filtered through glass filter G4. Caffeine was removed by shaking the filtrate 3 × with 1/2 vol. of $CHCl_3$.

Chromatography and determination of radioactivity. Following evaporation of $CHCl_3$, the residue was dissolved in H_2O and an aliquot chromatographed for 2 hr on F 254 silica gel TLC plates with $CHCl_3$–EtOH (9:1) as solvent. The caffeine spot, localized under UV light, was cut out and the amount of caffeine eluted (with H_2O) was determined spectrophotometrically at 271 nm. Radioactivity was measured by liquid scintillation counting using Whatman GF/C glass fibre filters in a 10 ml mixture of dimethyl-POPOP, PPO and toluene. Dpm values were calculated by the channel ratio. Distribution of radioactivity on TLC plates was initially monitored with a radiochromatogram scanner.

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[11] VIRTANEN, A. I. (1955) *Angew. Chem.* **67**, 381.
[12] KOGAN, L., DICARLO, F. J. and MAYNARD, W. E. (1953) *Anal. Chem.* **25**, 1118.