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CAFFEINE BIOSYNTHESIS IN *CAMELLIA SINENSIS*

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Key Word Index—*Camellia sinensis*; Theaceae; tea; biosynthesis; purine metabolism; 7-methylxanthine; theobromine; caffeine; adenine; guanine.

Abstract—The metabolism of adenine and guanine, relating to the biosynthesis of caffeine, in excised shoot tips of tea was studied with micromolar amounts of adenine-[8-^{14}C] or guanine-[8-^{14}C]. Among the presumed precursors of caffeine biosynthesis, adenine was the most effective, whereas guanine was the least effective. After administration of a 'pulse' of adenine-[8-^{14}C], almost all of the adenine-[^{14}C] supplied disappeared by 30 hr, and ^{14}C-labelled caffeine and RNA purine nucleotide (AMP and GMP) synthesis increased throughout the experimental period, whereas the radioactivities of free purine nucleotides, 7-methylxanthine and theobromine increased during the first 10 hr incubation period, followed by a steady decrease. By contrast, more than 45% of the guanine-[8-^{14}C] supplied remained unchanged even after a 120 hr period. The main products of guanine-[8-^{14}C] metabolism in tea shoot tips were guanine nucleotides, theobromine, caffeine and the GMP of RNA. The results support the hypothesis that the purine nucleotides are synthesized from adenine and guanine via the pathway of purine salvage. Adenylate is readily converted into other purine nucleotides, whereas the conversion rate of guanylate into other purine nucleotides is very low.

The results also support the view that 7-methylxanthine and theobromine are precursors of caffeine. For the origin of the purine ring in caffeine, purine nucleotides in the nucleotide pool rather than in nucleic acids are suggested.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is synthesized from the same precursors utilized for purine and methyl group synthesis in other systems [1–3]. Among methylated xanthines, 7-methylxanthine and theobromine (3,7-dimethylxanthine) have been reported to be precursors of caffeine [4–6]. *S*-Adenosylmethionine is the actual Me donor in the methylations of these methylxanthines [6–8]. However, neither the pathways leading to the formation of 7-methylxanthine, nor the relationship between purine metabolism and caffeine biosynthesis is understood.

In 1969, Inoue and Akiyama [9] found that adenine, guanine and hypoxanthine were all utilized in caffeine synthesis in tea plants; however, due to lack of information on the metabolism of purine bases in tea plants, they speculated that all these purines were converted via xanthine into caffeine. On the contrary, Ogutuga and Northcote [4] reported two possibilities for the origin of the purine ring in caffeine: one directly from xanthine and the other from the breakdown of nucleic acids, and postulated the latter the more likely according to isotope incorporation kinetics after continuous and pulse feeding of tea callus tissue with $^{14}CO_2$ and L-methionine-[Me-^{14}C]. This hypothesis has been supported by Konishi and Oishi [10], who found that xanthine was not converted into caffeine in tea plants. In contrast, based on isotope incorporation pattern after infiltrating coffee leaf disks simultaneously with L-methionine-[Me-^{14}C] and with presumed precursors, Looser *et al.* [5] proposed that the purine ring of caffeine is derived from free purine nucleotides, although they agreed with the hypothesis of Ogutuga and Northcote [4] that caffeine is synthesized from 7-methylxanthosine or from 7-methylxanthine via theobromine.

Previously, we revealed that tea plants can catabolize xanthine and hypoxanthine by the same pathways as purine catabolism in animals and that the formation of caffeine from hypoxanthine is a result of nucleotide synthesis via the pathway of purine salvage [11]. The synthesis of caffeine from adenine and guanine in tea plants has now been studied.

RESULTS

Feeding experiments with potential ^{14}C-labelled precursors

The synthesis of caffeine and related xanthines was investigated by feeding four shoot tips with presumed ^{14}C-labelled precursors (5 μCi in 0.5 ml of solution) for 1 hr and incubating the tips in water for 9 hr (Table 1). Almost all of the radioactive compounds supplied were taken up during the first 1 hr absorption period. L-Methionine-[Me-^{14}C] was used as the Me-group donor in caffeine biosynthesis and the radioactive purines were used as the possible precursors of purine ring of caffeine. Under these conditions, adenine-[8-^{14}C] was the best precursor for the synthesis of theobromine and

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Table 1. Incorporation of ^{14}C-labelled precursors into methylated xanthines, and AMP and GMP of RNA in tea shoot tips

Precursor	(cpm/shoot) 7-Methylxanthine	Theobromine	Caffeine	AMP	GMP
L-Methionine-[Me-^{14}C]	Trace	26100	56300	—	—
Hypoxanthine-[8-^{14}C]	Trace	19800	17800	880	16100
Adenine-[8-^{14}C]	40200	201000	116000	62400	44900
Guanine-[8-^{14}C]	Trace	14600	10200	Trace	49600

Each of four excised shoot tips (2.5–2.6 g fr wt) of 85-day-old seedlings was placed with its cut end in a small vial containing 5 μCi of the radioactive compound in 0.5 ml of solution for 1 hr, followed by incubation for a 9 hr period in a 50 ml conical flask, and was then processed as described in the Experimental.

caffeine, whereas significant amounts of radioactivity from L-methionine-[Me-^{14}C] were also incorporated into theobromine and caffeine. On the contrary, when tea shoot tips were fed with guanine-[8-^{14}C], only small amounts of radioactivity were incorporated into theobromine and caffeine. Although Ogutuga and Northcote [4] reported 7-methylxanthine as a product of L-methionine-[Me-^{14}C] metabolism in tea callus tissue, only in adenine-[8-^{14}C]-feeding experiments was there a significant incorporation of radioactivity into 7-methylxanthine.

The incorporation from ^{14}C-labelled purines into RNA purine nucleotides 9 hr after supplying radioactive purines to tea shoot tips is also shown in Table 1. Among the exogenous purines, adenine-[8-^{14}C] was best utilized for the synthesis of AMP and GMP of RNA. In contrast the incorporation of radioactivity from guanine-[8-^{14}C] into the AMP of RNA was almost nil, whereas a considerable amount of radioactivity from guanine-[8-^{14}C] was incorporated into the GMP of RNA.

Analysis of the metabolites produced from exogenous adenine and guanine

The radioactivity distribution between the compounds of the cold-acid soluble fraction was analysed 9 hr after supplying adenine-[8-^{14}C] or guanine-[8-^{14}C] to tea shoot tips. The acid-soluble fractions were subjected to 2-D PC by the systems described in the Experimental, and the radioactive areas on the chromatograms were located by autoradiography. Chromatographic analysis of the acid-soluble materials revealed that, in both cases, 7-methylxanthine, theobromine, caffeine and free purine nucleotides were all important products labelled with adenine-[8-^{14}C] and with guanine-[8-^{14}C]. In addition to these, there were also minor unidentified products. However, in neither case was the labelling of purine nucleosides (adenosine or guanosine), hypoxanthine and xanthine detected. There was also no radioactivity in compounds resulting from degradation of the purine ring (allantoic acid, allantoin, urea).

Subsequently, the purine nucleotides were eluted with water and analysed for radioactivity distribution. After hydrolysis of the pooled effluents from the chromatograms with 1 M HCl at 100° for 1 hr and rechromatography by the systems described in the Experimental, it was confirmed that 84 and 9% of the total nucleotide radioactivity derived from adenine-[8-^{14}C] were recovered in adenine and guanine respectively, with the remaining 4% and 3% in two unidentified compounds. By contrast more than 98% of the total nucleotide radioactivity derived from guanine-[8-^{14}C] was recovered in guanine, with the remainder in an unidentified compound.

Sequence of incorporation of radioactivity into products of adenine and guanine metabolism in tea shoot tips

The radioactivity distribution between the compounds of the cold-acid soluble fraction was analysed by feeding tea shoot tips with adenine-[8-^{14}C] (5 μCi in 0.5 ml of solution) or with guanine-[8-^{14}C] (5 μCi in 0.5 ml of solution) for 1 hr and then incubating the tips in water for various periods of time (Fig. 1). Almost all of the radioactive compounds supplied were taken up during the first 1 hr absorption period. When tea shoot tips were incubated with adenine-[8-^{14}C], the radioactivity of adenine-[8-^{14}C] supplied decreased during the first 10 hr incubation period, and only small amounts of radioactivity remained as unchanged adenine after a 30 hr period (Fig. 1a). The radioactivity of free purine nucleotides derived from adenine-[8-^{14}C] reached almost the same as that of adenine-[8-^{14}C] supplied within the first 1 hr absorption period and continued to rise during the first 10 hr incubation period, followed by a steady decrease, at first rapidly and then more slowly. A significant amount of radioactivity of 7-methylxanthine appeared during the first 10 hr incubation period; the value reached a maximum at 10 hr, followed by a steady decline. The radioactivity of theobromine reached a maximum value at 10 hr, and then decreased slowly, whereas the radioactivity of caffeine continued to increase steadily during the experimental periods, the value then reaching a maximum at 70 hr. Compared with the results from adenine-[8-^{14}C], the radioactivity of guanine-[8-^{14}C] supplied decreased slowly, and more than 45% of the ^{14}C absorbed still remained as unchanged guanine even after a 120 hr period (Fig. 1b). The radioactivity of free purine nucleotides derived from guanine-[8-^{14}C] appeared within the 1 hr absorption period, the value then reaching a maximum at 10 hr, followed by a steady decrease. The radioactivity of theobromine reached a maximum value at 30 hr, and then decreased, whereas the radioactivity of caffeine, after a lag period, increased steadily throughout the experimental period, the value reaching a maximum at 120 hr. The labelling of 7-methylxanthine was small and transient, disappearing 30 hr after feeding with guanine-[8-^{14}C].

Analyses were also conducted with the synthesis of RNA purine nucleotides (Fig. 2). When tea shoot tips were incubated with adenine-[8-^{14}C], radioactivity was rapidly incorporated into AMP and GMP of RNA (Fig. 2a). The incorporation of radioactive adenine into GMP was almost the same as that into AMP after a 20 hr period, even though there was a greater incorporation of radioactive adenine into AMP than into GMP during the first 10 hr incubation period. In both nucleotides,

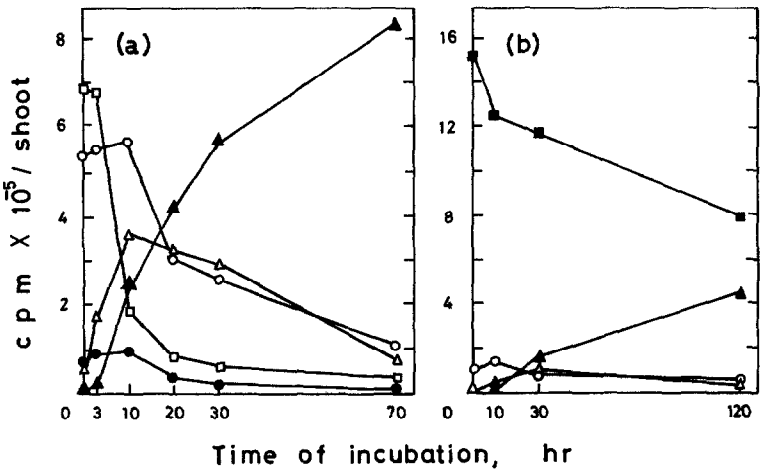

Fig. 1. Distribution of radioactivity among adenine (□), guanine (■), free purine nucleotides (○), 7-methylxanthine (●), theobromine (△) and caffeine (▲), when tea shoot tips were incubated with 5 μCi of adenine-[8-^{14}C] (a) or with 5 μCi of guanine-[8-^{14}C] (b). Groups of 4 excised shoot tips (2.5–2.6 g fr. wt) were each fed with 5 μCi of adenine-[8-^{14}C] or with 5 μCi of guanine-[8-^{14}C] within 1 hr and then incubated in H_2O in 50 ml flasks for various periods.

the radioactivities derived from adenine-[8-^{14}C] continued to rise steadily throughout the experimental period, the value then reaching a maximum at 70 hr. In contrast with the results with adenine-[8-^{14}C], almost all of the radioactivities in RNA derived from guanine-[8-^{14}C] were found only in GMP (Fig. 2b). Again, as described above, only very small amounts of radioactive guanine were incorporated into the AMP of RNA throughout the experimental period (less than 300 cpm shoot even after a 120 hr period). The radioactivity of GMP derived from radioactive guanine continued to increase steadily during the experimental periods and reached a maximum value at 120 hr.

DISCUSSION

The results presented in this paper support the suggestion that 7-methylxanthine and theobromine are precursors of caffeine. Although Ogutuga and Northcote [4] reported 7-methylxanthine as a product of L-methionine-[Me-^{14}C] metabolism in tea callus tissue, our results indicated that, in adenine-[8-^{14}C]-feeding experiments with tea shoot tips, there was a significant incorporation of radioactivity into 7-methylxanthine (Table 1).

Our results also argue for the synthesis of purine nucleotides from adenine and guanine via the pathway of purine salvage in tea shoot tips. There are three main possible routes whereby purine nucleotides are synthesized from free purines [12]. Among them, salvage pathways involving conversion of purines into nucleosides and of nucleosides into nucleotides, or involving conversion of adenine and guanine into hypoxanthine and xanthine and of hypoxanthine and xanthine into IMP and XMP appear to be little importance in tea shoot tips, because no labelling of purine nucleosides, hypoxanthine

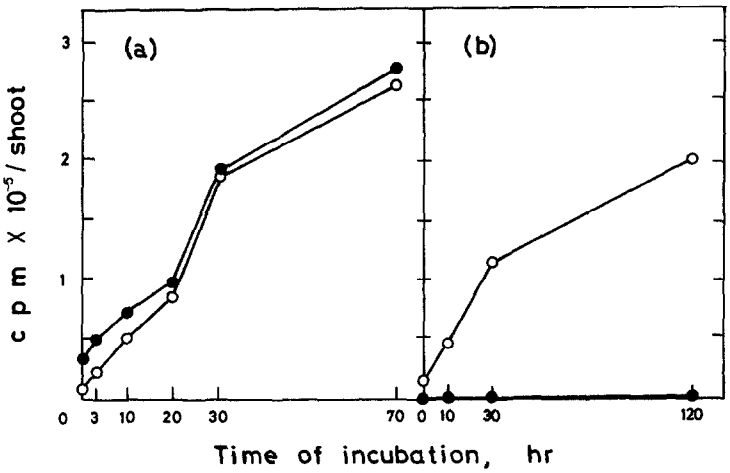

Fig. 2. Incorporation of radioactivity from adenine-[8-^{14}C] (a) and from guanine-[8-^{14}C] (b) into the AMP (●) and GMP (○) of RNA in tea shoot tips. Groups of four excised shoot tips (2.5–2.6 g fr. wt) were each fed with 5 μCi of adenine-[8-^{14}C] or with 5 μCi of guanine-[8-^{14}C] within 1 hr and then incubated in H_2O in 50 ml flasks for various periods.

and xanthine occurs. In contrast adenine and guanine can react directly with PRPP to yield AMP and GMP, respectively. These involve the two different enzymes, adenine phosphoribosyltransferase (EC 2.4.2.7) and hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8). Time-course analysis study (Figs. 1 and 2) indicates that the activity of the former enzyme is much higher than that of the latter in tea shoot tips.

The rapid incorporation of radioactive adenine into the GMP of RNA (Fig. 2a) suggests that adenylate is readily converted into other purine nucleotides in tea shoot tips. These processes probably involve the conversion of AMP into IMP catalysed by the enzyme, AMP deaminase (EC 3.5.4.6) [12]. The small incorporation of radioactive guanine into the AMP of RNA (Fig. 2b) indicates that the conversion of GMP into IMP catalysed by the enzyme, GMP reductase (EC 1.6.6.8) [12], is not an important reaction in tea shoot tips.

There may be two sources for the purine ring in caffeine [11]; the methylated nucleotides in nucleic acids [4] and the methylated nucleotides in the nucleotide pool [5]. Our results (Figs. 1 and 2) indicate the latter. In adenine-[8-^{14}C]-feeding experiments, the former cannot account for the decrease of radioactivities in 7-methylxanthine and theobromine between the 20 hr and 70 hr incubation periods. Since free purine nucleotides are synthesized from adenine as the first products of adenine-[8-^{14}C] metabolism in tea shoot tips, the decrease of radioactivities in adenine and free nucleotides may also result in the subsequent decrease of radioactivities in 7-methylxanthine and theobromine. The continuous increase of radioactivity in caffeine is accompanied by the decrease of radioactivities in these precursors.

Our results also suggest that both adenine and guanine nucleotides can serve as the precursors of caffeine biosynthesis. Although, from the small incorporation of radioactive guanine into the AMP of RNA (Fig. 2b), guanine nucleotides appear to be more likely precursors of caffeine biosynthesis, this cannot account for a much greater incorporation of radioactive adenine into caffeine (Fig. 1a). At present, one cannot decide which nucleotides play the most important roles in caffeine biosynthesis.

EXPERIMENTAL

Materials. Adenine-[8-^{14}C] (54 mCi/mmol) and guanine-[8-^{14}C] (56 mCi/mmol) were purchased from The Radiochemical Center, Amersham, U.K. Hypoxanthine-[8-^{14}C] (47 mCi/mmol) and L-methionine-[Me-^{14}C] (53 mCi/mmol) were purchased from Le Commissariat á l'Énergie Atomique, Paris, France. In all feeding expts radioactive compounds were dil with H_2O to give 10 μCi in 1 ml of soln.

Plant material. 85-day-old tea seedlings (*Camellia sinensis* L.) grown in daylight in a greenhouse maintained at 25° during the day and at 15° at night were used as described in refs [8, 11].

Feeding experiments. The expts were carried out at 25° in daylight in a greenhouse. 4 shoot tips (2.5–2.6 g fr wt), comprising the 3 developed leaves, were used [8, 11]. Cut ends of excised shoot tips were placed in small vials containing the required quantity of radioactive compound. After absorption of the radioactive compound, tips were transferred to H_2O in 50-ml conical flasks. At the end of the incubation periods, tips were frozen until required.

Extraction and preparation of acid-soluble materials and RNA nucleotides. The frozen shoot tips were cut into small pieces, and ground with cold 20 ml of 0.2 M $HClO_4$ (0.6 M $HClO_4$ for extraction of free nucleotides) and washed sea sand in a chilled mortar. After centrifugation the residue was re-extracted with 10 ml of cold 0.2 M $HClO_4$ (0.6 M $HClO_4$ for extraction of free nucleotides) and the washings were combined with the supernatant. The combined supernatants were adjusted to pH 6.5 with solid $KHCO_3$ and the $KClO_4$ removed by centrifugation. The neutralized supernatants of the acid-soluble material were applied to a cationic resin column (Amberlite IR-120, H^+ form) and the adsorbed fraction (amino acids, bases) eluted with 2 M NH_4OH. The effluent was dried *in vacuo* and dissolved in 0.5 ml H_2O. Acid-insoluble residue was successively washed with 2 × 40 ml of 95% EtOH, 40 ml of EtOH–$CHCl_3$ (3:1), 3 × 30 ml of Et_2O, and finally dried *in vacuo*. The dry powder was subjected to alkaline hydrolysis of RNA with 0.5 M KOH at 37° for 20 hr [13].

Chromatography and determination of radioactivity. Neutralized supernatants of acid-soluble materials and the 2 M NH_4OH eluates from the Amberlite IR-120 (H^+ form) resin were subjected to 2-D autoradiography to characterize the ^{14}C-labelled products and to assay their radioactivities. PC was carried out by ascending development on Whatman No. 1, using the following systems: (1) *n*-BuOH–HOAc–H_2O (4:1:1) followed by EtOH–HOAc–H_2O (81:5:14) [14]; (2) EtOH–HOAc–H_2O (81:5:14) followed by Py–NH_4OH (47:3) [15]. Authentic materials were co-chromatographed on each sheet. Radioactive areas on the chromatograms were located by autoradiography. Caffeine and authentic bases, nucleosides and nucleotides were detected by UV quenching (254 nm) and the authentic allantoin, allantoic acid and urea were detected with Ehrlich reagent. By using PC either in solvents (1) or (2), radioactivity of the ^{14}C-labelled products was assayed in a liquid-scintillation counter as described in refs [8, 11]. The alkaline hydrolysates of RNA, after removal of DNA, protein, cellulose, sea sand and $KClO_4$ by centrifugation, were separated by chromatography on a column of Dowex 1 (×2; formate form; 200–400 mesh) and elution by a gradient of 1–4 M formic acid [14]. The effluents were collected in 3 ml fractions and the E_{260} of each fraction was measured. The identity of the nucleotides was established by their elution positions from the resin and by PC co-chromatography on Whatman No. 1 with authentic nucleotides in solvents (3) *iso*PrOH-conc HCl–H_2O (14:3:3), (4) MeOH–conc HCl–H_2O (7:2:1) and (5) *iso*butyric acid-0.5 M NH_4OH (5:3). Detection of the UV-absorbing areas on the PC were described above. For a quantitative assay of the ^{14}C-labelled AMP and GMP, UV-absorbing fractions corresponding to AMP and GMP were collected from the column. They were then dried *in vacuo* and dissolved in 0.5 ml H_2O. After rechromatography on Whatman 3MM paper in solvent (3) or (5), the positions of the UV-absorbing spots on the papers corresponding to AMP and GMP were marked in pencil, cut from the papers, and their radioactivities measured in a liquid-scintillation counter as described in refs [8, 11].

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