Metabolism of Methionine and Biosynthesis of Caffeine in the Tea Plant (*Camellia sinensis* L.)

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(Received 14 April 1976)

1. Caffeine biosynthesis was studied by following the incorporation of ^{14}C into the products of L-[Me- ^{14}C]methionine metabolism in tea shoot tips. 2. After administration of a 'pulse' of L-[Me- ^{14}C]methionine, almost all of the L-[Me- ^{14}C]methionine supplied disappeared within 1 h, and ^{14}C -labelled caffeine synthesis increased throughout the experimental periods, whereas the radioactivities of an unknown compound and theobromine were highest at 3 h after the uptake of L-[Me- ^{14}C]methionine, followed by a steady decrease. There was also slight incorporation of the label into 7-methylxanthine, serine, glutamate and aspartate, disappearing by 36 h after the absorption of L-[Me- ^{14}C]methionine. 3. The radioactivities of nucleic acids derived from L-[Me- ^{14}C]methionine increased rapidly during the first 12 h incubation period and then decreased steadily. Sedimentation analysis of nucleic acids by sucrose-gradient centrifugation showed that methylation of nucleic acids in tea shoot tips occurred mainly in the tRNA fraction. The main product among the methylated bases in tea shoot tips was identified as 1-methyladenine. 4. The results indicated that the purine ring in caffeine is derived from the purine nucleotides in the nucleotide pool rather than in nucleic acids. A metabolic scheme to show the production of caffeine and related methylxanthines from the nucleotides in tea plants is discussed.

Much work on caffeine biosynthesis, both in tea (*Camellia sinensis* L.) and in coffee (*Coffea arabica* L.) plants, has been conducted on methylation with methionine (Anderson & Gibbs, 1962; Inoue & Adachi, 1962; Ogotuga & Northcote, 1970a,b; Keller *et al.*, 1972; Suzuki, 1972; Looser *et al.*, 1974). However, neither the metabolism of methionine in these plants nor the relationship that it might have to caffeine biosynthesis is understood. We have revealed that S-adenosylmethionine acts as the actual methyl donor in the methylations of caffeine precursors from methionine (Suzuki & Takahashi, 1975b).

Suzuki (1973) reported that there was heavy labelling of theobromine, caffeine and an unknown compound within 1 h after the feeding of tea shoot tips with L-[Me- ^{14}C]methionine. Looser *et al.* (1974) also reported that a high percentage of methionine is quickly converted into an unknown compound after infiltrating coffee-leaf discs with L-[Me- ^{14}C]methionine. These facts suggest a rapid turnover rate of methionine in these plants.

There are two possible sources of the purine ring in caffeine (Suzuki & Takahashi, 1975a): the methylated nucleotides in nucleic acids (Ogotuga & Northcote, 1970b) and the methylated purine nucleotides in the nucleotide pool (Looser *et al.*, 1974; Suzuki & Taka-

hashi, 1976a). On the basis of a study of isotope-incorporation kinetics after continuous and pulse feeding of tea callus tissue with L-[Me- ^{14}C]methionine and $^{14}\text{CO}_2$, Ogotuga & Northcote (1970b) demonstrated the importance of methylation of nucleic acids in the formation of methylated purine nucleotides, suspected precursors of caffeine. In contrast, we have postulated that the precursors are the purine nucleotides in the nucleotide pool rather than in nucleic acids, on the basis of isotope-incorporation kinetics after pulse feeding of tea shoot tips with [8- ^{14}C]adenine and [8- ^{14}C]guanine (Suzuki & Takahashi, 1976a). Therefore we have now further studied the methylation of nucleic acids *in vivo* and *in vitro*, and the relationship that it might have to caffeine biosynthesis in tea plants.

The present paper describes experiments in which L-[Me- ^{14}C]methionine was fed to excised tea shoot tips, and the subsequent fate of this compound was then investigated. The α -carbon of glycine and β -carbon of serine are also important methyl donors in caffeine biosynthesis (Anderson & Gibbs, 1962), and glycine is the established precursor of purine biosynthesis *de novo*. Thus in some experiments [2- ^{14}C]glycine, [1- ^{14}C]glycine and L-[3- ^{14}C]serine were also used to estimate the turnover rate of these amino acids in amino acid metabolism and of nucleic acid bases in tea shoot tips. Results are discussed in relation to the possible pathways for methionine metabolism and for caffeine biosynthesis in tea plants.

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Materials and Methods

Chemicals

Materials were obtained from the following sources: L-[Me- 14 C]methionine (53 mCi/mmol) and [2- 14 C]glycine (36.7 mCi/mmol) from Le Commissariat à l'Énergie Atomique, Paris, France; L-[3- 14 C]-serine (38.9 mCi/mmol) and [1- 14 C]glycine (19.4 mCi/mmol) from Daiichi Pure Chemicals Co., Tokyo, Japan; 7-methylxanthine, prepared from XMP as described by Jones & Robins (1963); XMP, 1-methyladenine, 2-methyladenine hemi-sulphate and 7-methylguanine from Sigma Chemical Co., St. Louis, MO, U.S.A.; brewer's-yeast tRNA from Boehringer, Mannheim, Germany; Polyclar AT (insoluble polyvinylpyrrolidone) from General Aniline and Film Corp., Dyestuff and Chemical Division, New York, NY, U.S.A.

Plants and feeding experiments

The plant material and the methods of the feeding experiments were as described by Suzuki (1973). Tea shoot tips (comprising the bud, three developed leaves and the included stem), or tea leaves were harvested from 80–90-day-old seedlings except as otherwise noted.

Extraction and preparation of acid-soluble materials, lipid and RNA nucleotides

Extraction and preparation were as described by Suzuki (1973), except for the following modifications. After re-extraction with 5 ml of cold 0.2M-HClO₄, the acid-insoluble residue was washed with 2 × 10 ml of cold water, and the washings were combined with the supernatant fluids.

Radioactivity of acid-soluble materials and lipid fraction was determined in a Kobe Kogyo Corp. type PC-26 gas-flow counter as described by Suzuki (1973).

Radioautography of 14 C-labelled amino acids and methylated xanthines

The acid-soluble materials, after neutralization and removal of KClO₄, were passed through a column (1.2 cm × 15 cm) of the sulphonic resin Amberlite IR-120 (H⁺ form) and eluted with 2M-NH₃. This eluate contained amino acids and methylated xanthines. The methods used for separation of the 2M-NH₃ eluate by paper chromatography and for location by radioautography were as described by Suzuki (1973). Radioactivity of the 14 C-labelled products was assayed in a Beckman LS-100 liquid-scintillation counter as described by Suzuki (1973).

Column and paper chromatography of alkaline hydrolysates of RNA

The column-chromatographic methods used for separation of alkaline hydrolysates of RNA and for measurements of the E_{260} were as described by Suzuki (1973). The methods for determining radioactivities

of the 14 C-labelled AMP and GMP were as described by Suzuki & Takahashi (1975a). Radioactivities were measured in a Beckman LS-100 liquid-scintillation counter as described above.

Extraction and purification of nucleic acids

A modification of the method of Bhattacharyya & Ghosh (1968) was used. The frozen shoot tips were cut into small pieces, and ground with 50–60 ml of cold 0.2M-Tris/HCl buffer, pH 7.8, containing 0.005% EDTA and 1% (w/v) sodium dodecyl sulphate, and an equal weight of washed Polyclar AT and about 2 g of washed sea sand in a pre-chilled mortar. The homogenates were then squeezed through cheesecloth. The filtrate was transferred to a glass-stoppered bottle and stirred for 2 h at 20°C with an equal volume of water-saturated phenol. The phenol and aqueous phase and the interphase layer were separated by centrifugation at 1000g for 20 min at 4°C. The upper aqueous phase was removed by pipette and the phenol phase (plus interphase) was washed with an equal volume of 0.2M-Tris/HCl buffer, pH 7.8. The aqueous phase and the wash were combined, the nucleic acid was precipitated by adding 2.2 vol. of 2% (w/v) potassium acetate and 2 vol. of 96% (w/v) ethanol and the mixture was kept at –10°C overnight.

The nucleic acid precipitate was collected by centrifugation at 3000g for 20 min at 4°C and the precipitate was washed with 50, 70 and 96% (w/v) ethanol followed by diethyl ether before being dissolved in 0.2M-Tris/HCl buffer, pH 7.8. The protein that remained in the preparation was removed by shaking the nucleic acid solution vigorously with an equal volume of a mixture of chloroform/octan-1-ol (8:1, v/v), followed by centrifugation at 3000g for 20 min (Sevag *et al.*, 1938). This process was repeated until no white gel was formed on the addition of the chloroform/octan-1-ol mixture. The nucleic acid solution was then dialysed for 24 h at 0–4°C against four changes of water (each 1 litre). The dialysed solution was used for determination of spectral and sedimentation characteristics of nucleic acids. The spectrophotometric readings were made with a Shimadzu double-beam UV-200 spectrophotometer.

Radioactivity of nucleic acid was determined as follows. The dialysed solution was introduced on to a Whatman glass-fibre paper (GF/C) and dried under an i.r. lamp. To this was added 5 ml of a toluene-based scintillator solution in a counting vial. This solution contained 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene. Radioactivity was measured in a Beckman LS-100 liquid-scintillation counter as described above. Measurements were corrected for background counts.

Sucrose-gradient centrifugation

Nucleic acid samples (0.3 ml) were layered over linear 5–20% (w/v) sucrose-density gradients con-

taining 0.05 M-Tris/HCl, pH 7.4, 0.5 M-NaCl and 3 mM-EDTA. Gradients were centrifuged for 13 h at 55000g in a Hitachi RPS 65TA rotor at 4°C. Six drops were collected in each fraction. The E_{260} of each fraction was measured with a Shimadzu QV-50 spectrophotometer. Radioactivity was assayed by mixing each of the fractions (approx. 0.17 ml) with 10 ml of Triton X-100/the toluene-based scintillator solution used above (1:2, v/v) and counting in a Beckman LS-100 liquid-scintillation counter. Measurements were corrected for background counts.

Acid hydrolysis of nucleic acids

The nucleic acid was precipitated from the dialysed solution with 2.2 vol. of 2% (v/v) potassium acetate and 2 vol. of 96% (v/v) ethanol overnight at -10°C. The precipitate was centrifuged (3000g; 20 min), washed with 96% (v/v) ethanol followed by diethyl ether and finally dried *in vacuo*. A sample (4 mg) of dried nucleic acid was introduced into a small glass tube (0.5 cm × 4 cm) and 0.2 ml of 9M-HClO₄ was added before the tube was sealed. Hydrolysis was then carried out to yield free bases at 100°C for 90 min (Wyatt, 1952).

Chromatography of nucleic acid bases

The sealed tubes were opened, the hydrolysate was diluted with water and then neutralized with 0.5M-KOH. After centrifugation (7000g; 15 min), the precipitate was washed once with 0.1M-HCl, then discarded. The supernatants were combined, evaporated *in vacuo* and redissolved in 0.05 ml of 0.1M-HCl.

All chromatograms were run in the ascending direction on Whatman 3MM paper, with the following solvent systems: (A) propan-2-ol/12M-HCl/water (85:21:18, by vol.); (B) butan-1-ol/aq. 0.6M-NH₃ (6:1, v/v); (C) propan-1-ol/aq. 15M-NH₃/water (70:1:29, by vol.). After chromatography, the positions of the u.v.-absorbing spots (at 253.7 nm) corresponding to the authentic purines were marked in pencil, papers were cut into pieces (4 cm wide × 1 cm), transferred to vials with 5 ml of the toluene-based scintillator solution used above and their radioactivities were measured in a Beckman LS-100 liquid-scintillation counter, as described above.

Results

Metabolism of L-[Me-¹⁴C]methionine, [2-¹⁴C]glycine and L-[3-¹⁴C]serine by excised tea shoot tips

In these experiments, samples of tea shoot tips were incubated in water for 1 or 2 h after the uptake of the radioactive compounds (Table 1). Approx. 44, 52 and 41% of the ¹⁴C supplied were recovered in the acid-soluble fractions of the samples fed with L-[Me-¹⁴C]methionine, [2-¹⁴C]glycine and L-[3-¹⁴C]serine respectively.

When tea shoot tips were incubated in water for 1 h after the uptake of L-[Me-¹⁴C]methionine, only a trace of radioactivity was detected in methionine, and approx. 17% of the ¹⁴C incorporated into the 2M-NH₃ eluate was present in caffeine and related methylxanthines. More than 82% of the ¹⁴C incorporated into the 2M-NH₃ eluate was found in unknown compounds X and Y. Chromatographic analysis of these unknown compounds showed that they were not methylated xanthines (Fig. 1). Smaller amounts of radioactivity were also found in serine, glutamate and aspartate. In contrast, approx. 24 and 29% of the ¹⁴C recovered in the 2M-NH₃ eluates of the samples fed with [2-¹⁴C]glycine and L-[3-¹⁴C]serine were found in glycine and serine respectively. In both cases, glutamate, aspartate, glutamine, alanine, γ -aminobutyrate, theobromine and caffeine are the major products of [2-¹⁴C]glycine and L-[3-¹⁴C]serine metabolism, and small amounts of radioactivity were found in β -alanine and 7-methylxanthine. In addition, in the [2-¹⁴C]glycine-feeding experiments, a large amount of radioactivity was also incorporated into serine and smaller amounts of radioactivity were found in cysteine and cystine.

Fig. 1. Tracing of a two-dimensional radioautogram of radioactive products of amino acids and methylated xanthines from tea shoot tips 1 h after the absorption of L-[Me-¹⁴C]-methionine (10 μ Ci in 1 ml of solution)

The 2M-NH₃ eluate from a column of Amberlite IR-120 (H⁺ form) was subjected to two-dimensional ascending chromatography on Whatman no. 1 paper, with phenol/water (4:1, v/v) in direction 1 and butan-1-ol/acetic acid/water (4:1:1, by vol.) in direction 2. The hatched spots represent the radioactive products. Theanine (γ -glutamyl-ethylamide) and γ -glutamylmethylamide are shown as reference compounds. Abbreviations: 7-MX, 7-methylxanthine; Tb, theobromine; Ca, caffeine; γ -GM, γ -glutamylmethylamide; Thea, theanine; X and Y, unknown substances.

Table 1. *Metabolism of L-[Me-¹⁴C]methionine, [2-¹⁴C]glycine and L-[3-¹⁴C]serine by excised tea shoot tips*

Each of four excised tea shoot tips (2.5–2.6 g fresh wt.) was placed with its cut end in a small vial containing 10 μ Ci of the radioactive compound (specific radioactivity 30 mCi/mmol) in 1.0 ml of solution for 2 h, followed by incubation, for the times indicated, in water in a 50 ml conical flask and then processed as described in the Materials and Methods section. n.d., Not detectable. tr., Trace.

Fraction	L-[Me- ¹⁴ C]Methionine, 1 h		[2- ¹⁴ C]Glycine, 2 h		L-[3- ¹⁴ C]Serine, 2 h	
	Radioactivity (c.p.m.)	% of ¹⁴ C incorporated	Radioactivity (c.p.m.)	% of ¹⁴ C incorporated	Radioactivity (c.p.m.)	% of ¹⁴ C incorporated
Acid-soluble	1444000	43.7	1713000	51.9	1353000	41.0
Lipids	106000	3.2	120000	3.6	150000	4.5
Others*	1750000	53.0	1467000	44.5	1797000	54.5
Total ¹⁴ C absorbed	3300000	—	3300000	—	3300000	—
Radioactivity (c.p.m./shoot)						
Compounds analysed						
Methionine	tr.	(—)†	n.d.	(—)	n.d.	(—)
Glycine	n.d.	(—)	310400	(23.7)	n.d.	(—)
Serine	11800	(1.0)	218000	(16.6)	282000	(29.0)
Aspartic acid	1400	(0.1)	114400	(8.7)	148400	(15.3)
Glutamic acid	2000	(0.2)	206200	(15.7)	266600	(27.5)
Glutamine	n.d.	(—)	69000	(5.3)	86400	(8.9)
Alanine	n.d.	(—)	34000	(2.6)	38800	(4.0)
β -Alanine	n.d.	(—)	5000	(0.4)	6800	(0.7)
γ -Aminobutyric acid	n.d.	(—)	30400	(2.3)	64200	(6.6)
7-Methylxanthine	7200	(0.6)	17200	(1.3)	tr.	(—)
Theobromine	131600	(11.2)	219000	(16.7)	46400	(4.8)
Caffeine	54600	(4.7)	59800	(4.6)	19000	(2.0)
Others	965200	(82.2)	28000	(2.1)‡	12400	(1.3)
Total ¹⁴ C incorporated into amino acids and bases	1173800	(100)	1311400	(100)	971000	(100)

* Values for 'others' were obtained by subtracting the radioactivity in the remainder of the fractions from the radioactivity supplied.

† Numbers in parentheses represent % of the total radioactivity of the 2M-NH₃ eluate from a column of Amberlite IR-120 (H⁺ form).

‡ Includes 0.8 and 0.4% of cysteine and cystine respectively.

Sequence of incorporation of radioactivity into products of L-[Me-¹⁴C]methionine metabolism in tea shoot tips

The time-course for incorporation of radioactivity into products of L-[Me-¹⁴C]methionine metabolism was investigated by feeding tea shoot tips with L-[Me-¹⁴C]methionine within 1 h and incubating the tips in water for various periods (Table 2). The radioactivity of the L-[Me-¹⁴C]methionine supplied was completely converted into its metabolites during the first 4 h experimental period (1 h of absorption plus 3 h of incubation). The radioactivities of theobromine and the unknown compound X were highest at 3 h after the uptake of L-[Me-¹⁴C]methionine, and then decreased steadily. In contrast, the radioactivity of caffeine increased steadily and reached a maximum at 108 h. The labelling of 7-methylxanthine and other compounds (unknown compound Y, serine, glutamate and aspartate) was slight and transient, dis-

appearing 12 and 36 h respectively, after the feeding with L-[Me-¹⁴C]methionine.

Incorporation of L-[Me-¹⁴C]methionine into nucleic acids and of [1-¹⁴C]glycine into AMP and GMP of RNA in tea shoot tips

Since the importance of methylation of nucleic acids for the formation of methylated purine nucleotides, suspected precursors of caffeine, in caffeine biosynthesis has been demonstrated by Ogutuga & Northcote (1970b), it was decided to examine the relationship that it might have to caffeine biosynthesis in tea shoot tips.

The nucleic acids obtained in these experiments had a maximum extinction at 258–260 nm, and a minimum at 230–232 nm, at pH 5.7. Both $E_{\text{max.}}/E_{\text{min.}}$ ratio and $E_{\text{max.}}/E_{280}$ ratio in all the experiments were 1.80–1.90. The radioactivity of nucleic acids increased rapidly during the first 4 h experimental period and

Table 2. Sequence of incorporation of ^{14}C into products of L-[Me- ^{14}C]methionine metabolism in tea shoot tips

Batches of four excised shoot tips (2.5–2.6 g fresh wt.) were each fed with $5\mu\text{Ci}$ of L-[Me- ^{14}C]methionine (53 mCi/mmol) within 1 h and then incubated in water in 50 ml conical flasks for various periods. n.d., Not detectable. tr., Trace.

Time of incubation (h) ...	Radioactivity (c.p.m./shoot)			
	3	12	36	108
Methionine	n.d.	n.d.	n.d.	n.d.
7-Methylxanthine	tr.	n.d.	n.d.	n.d.
Theobromine	55 900	24 700	5400	tr.
Caffeine	31 700	61 600	88 600	89 300
Unknown X	203 000	140 000	98 000	41 000
Others*	6000	tr.	n.d.	n.d.

* Includes unknown Y, serine, glutamate and aspartate.

Fig. 2. Incorporation of radioactivity from (a) L-[Me- ^{14}C]methionine into nucleic acids and (b) [1- ^{14}C]glycine into AMP (○) and GMP (●) of RNA in tea shoot tips

Batches of four excised shoot tips (2.5–2.6 g fresh wt.) were each fed with $5\mu\text{Ci}$ of L-[Me- ^{14}C]methionine (53 mCi/mmol) or with $5\mu\text{Ci}$ of [1- ^{14}C]glycine (19 mCi/mmol) within 1 h and then incubated in water in 50 ml conical flasks for various periods.

reached a maximum at 12 h after the uptake of L-[Me- ^{14}C]methionine, followed by a steady decrease (Fig. 2a).

The turnover of AMP and GMP of RNA derived from purine biosynthesis *de novo* was also investigated by feeding tea shoot tips with $5\mu\text{Ci}$ of [1- ^{14}C]glycine (Fig. 2b). The radioactivities of AMP and GMP of RNA derived from [1- ^{14}C]glycine were increased during the first 12 h incubation period, followed by a steady but a rather slow decrease compared with the radioactivity of nucleic acids derived from L-[Me- ^{14}C]methionine. In addition, by paper chromatography and radioautography of the amino acid and methylated xanthine fraction, it was confirmed that almost all of the [1- ^{14}C]glycine supplied was metabolized by tea shoot tips during the first 12 h incubation period. No labelling of glycine was detected in the 36 and 108 h samples.

Examination of the radioactivity distribution in the nucleic acids in tea shoot tips after the feeding with L-[Me- ^{14}C]methionine

The radioactivity distribution in the nucleic acids methylated *in vivo* was investigated by feeding tea shoot tips with L-[Me- ^{14}C]methionine for 1 h and then incubating the tips in water for a 9 h period, during which time considerable methylation of nucleic acids in the tips was shown to occur (Fig. 2a). The nucleic acids were extracted and purified as described above, and then analysed for radioactivity distribution (Fig. 3). Almost all of the radioactivity in the nucleic acids derived from L-[Me- ^{14}C]methionine was detected in the tRNA fraction.

Characterization of the bases methylated *in vivo* in tea shoot tips

After acid hydrolysis of nucleic acids, obtained as

described above, the radioactivity distribution between the bases methylated in tea shoot tips was analysed by paper chromatography on Whatman 3MM paper in each of the systems described in the Materials and Methods section (Table 3). Three major peaks of radioactivity were detected on a paper chromatogram in solvent (A). Among them, the most major radioactive peak was identified as 1-methyladenine. By using paper chromatography in either solvent (B) or (C), 1-methyladenine was well separated from 2-methyladenine and 7-methylguanine, and in both cases the labelling of 1-methyladenine was confirmed. In contrast, two other

major radioactive peaks, which were well separated from authentic 7-methylguanine on a paper chromatogram in solvent (A), have not yet been identified.

Discussion

By using detached parts of coffee and tea plants (Looser *et al.*, 1974; Suzuki & Takahashi, 1976a) or callus-tissue culture of tea plants (Ogutuga & Northcote, 1970b) or cell-free extracts of tea leaves (Suzuki & Takahashi, 1975b), it has been shown that 7-methylxanthine and theobromine are precursors of caffeine. The present and the following paper (Suzuki & Takahashi, 1976b) describe a further study of caffeine biosynthesis associated with methionine metabolism and nucleic acid methylation *in vivo* and *in vitro*; the results presented support the above-mentioned demonstration.

Under the conditions described in Table 1, [2-¹⁴C]-glycine was a more effective precursor of 7-methylxanthine biosynthesis than was L-[Me-¹⁴C]methionine. This is comparable with that observed in tea shoot tips fed with [8-¹⁴C]adenine and L-[Me-¹⁴C]methionine (Suzuki & Takahashi, 1976a), in that adenine is a more effective precursor in biosynthesis of caffeine and related methylxanthines. The synthesis of the methylxanthines from [2-¹⁴C]glycine and [8-¹⁴C]adenine is probably due to active incorporation of radioactive purine nucleotides (synthesized from glycine by the pathways of purine biosynthesis *de novo* or from adenine by the pathway of purine salvage) into the purine ring in the methylxanthines, whereas the radioactivities derived from L-[Me-¹⁴C]methionine are exclusively due to the methylation from methionine (Anderson & Gibbs, 1962). As for incorporation from [2-¹⁴C]glycine, the

Fig. 3. Sucrose-density-gradient sedimentation profile of nucleic acids prepared from tea shoot tips (4g fresh wt.) labelled with 5 μ Ci of L-[Me-¹⁴C]methionine (53 mCi/mmol) for 10h

Brewer's-yeast tRNA was run in parallel to the samples (0.3ml) containing tea shoot nucleic acids as the tRNA marker (arrow). For details see the text. ●, E_{260} ; ○, ¹⁴C radioactivity (c.p.m.).

Table 3. Identification of the bases of nucleic acids from tea shoot tips (4g fresh wt.) labelled with 5 μ Ci of L-[Me-¹⁴C]methionine (53 mCi/mmol) for 10h

The R_F values were obtained by ascending chromatography on Whatman 3MM paper, with the solvent systems as described in the Materials and Methods section.

	Relative methylation (%)	Solvent ...	R_F values		
			A	B	C
Authentic bases					
Adenine	—		0.28	0.47	0.46
Guanine	—		0.16	0.18	0.34
1-Methyladenine	—		0.30	0.30	0.40
2-Methyladenine	—		0.31	0.58	0.49
7-Methylguanine	—		0.26	0.21	0.38
Bases methylated <i>in vivo</i>					
1	100		0.30	0.30	0.40
2	30		0.18	—	—
3	60		0.48	—	—
4	14		0.64	—	—
5	8		0.76	—	—
6	7		0.88	—	—

radioactivity may also be derived from the α -carbon atom of glycine via the C_1 pathways. The radioactivities derived from L-[3- 14 C]serine may be mainly due to the methylation via the C_1 pathways.

Although a rapid turnover rate of glycine in tea shoot tips was demonstrated by Roberts & Sanderson (1966), the results (Tables 1 and 2) suggest that the turnover rate of methionine in tea shoot tips is much more rapid than that of glycine and serine, and that the major labelled product of L-[Me- 14 C]-methionine metabolism in tea shoot tips is a relatively metabolically stable unknown compound rather than caffeine and related methylxanthines.

Besides the labelling of these major products (an unknown compound X, caffeine and theobromine), labelling of serine, glutamate and aspartate occurred in tea shoot tips as a result of feeding them with adequate amounts of L-[Me- 14 C]methionine (Table 1). Phosphatidylcholine or choline might be produced from phosphatidylethanolamine by methylation from S-adenosylmethionine (Van den Bosch, 1974). In

plants, Schaeffer & Sharpe (1971) reported incorporation of L-[Me- 14 C]methionine into phosphatidylcholine in tobacco plants. The present studies clearly show the incorporation of L-[Me- 14 C]methionine into lipid materials (Table 1). Further, glutamate and aspartate were shown to be the major products of L-[3- 14 C]serine metabolism in tea shoot tips. Thus labelling of serine, glutamate and aspartate could occur if the labelled choline produced from L-[Me- 14 C]methionine was further metabolized via betaine and sarcosine to formaldehyde (Cantoni, 1965). The formation of serine from formaldehyde and glycine in higher plants is now well characterized (Cossins & Sinha, 1966; Shah & Cossins, 1970).

A speculation that accords with these observations has been outlined (Scheme 1). Thus caffeine and related methylxanthines could be entirely formed from L-[Me- 14 C]methionine via S-adenosylmethionine. The labelling of glutamate, aspartate and alanine that occurred in tea shoot tips as a result of feeding them with [2- 14 C]glycine and L-[3- 14 C]serine (Table 1)

Scheme 1. Metabolism of L-[Me- 14 C]methionine, [2- 14 C]glycine and L-[3- 14 C]serine in tea shoot tips

The numbering (subscripts in parentheses) of the C atoms in each compound refers to the C atoms of the glycine or serine from which they are originally derived.

might be ascribed to the formation of the labelled pyruvate from radioactive serine by the action of serine dehydratase (Greenberg, 1962; Hill & Rogers, 1972). Further, labelling of glutamate and γ -aminobutyrate might occur as a result of the labelled glutamate formation from radioactive pyruvate via the tricarboxylic acid cycle. Finally, the labelling of lipid materials (Table 1) might be accounted for the formation of the radioactive acetyl-CoA via pyruvate from [2- 14 C]glycine and [3- 14 C]serine.

There may be two sources for the purine ring in caffeine: the methylated nucleotides in nucleic acids (Ogutuga & Northcote, 1970b) or the methylated nucleotides in the nucleotide pool (Looser *et al.*, 1974; Suzuki & Takahashi, 1976a). The results of the present experiments support the latter. The increase of radioactivity in caffeine derived from L-[Me- 14 C]methionine may surely be ascribed to the decrease of radioactivities in 7-methylxanthine and theobromine, suspected precursors of caffeine (Table 2). The unknown compounds X and Y appear not to be precursors of caffeine, because they are not methylxanthines (Fig. 1). There was also only a slight increase of radioactivity in caffeine 36 h after the uptake

of L-[Me- 14 C]methionine, whereas a considerable amount of radioactivity in the unknown compound X disappeared between the 36 and 108 h incubation periods. The low rate of incorporation of radioactive methionine into 7-methylxanthine compared with that into theobromine may surely be related to the rapid turnover rate of 7-methylxanthine as well as that of methionine. These observations are consistent with the results of degradation experiments done in coffee leaves by Anderson & Gibbs (1962), and with those done on caffeine biosynthesis by using tea leaf extracts (Suzuki & Takahashi, 1975b).

These speculations, based on methionine metabolism and nucleic acid methylation in tea shoot tips, are therefore contradictory to those reported by Ogutuga & Northcote (1970b). Our present results (Fig. 3 and Table 3) clearly show that the methylation of nucleic acids in tea shoot tips occurs mainly in N-1 of adenine in tRNA. No labelling of 7-methylguanine was detected in the amino acid and methylated xanthine fraction (Fig. 1). It is not known whether these differences may be because of the events that occurred in tea callus-tissue culture or excised shoot tips of tea plants. If so, it may be that both types of

Scheme 2. Suggested routes of the biosynthesis of caffeine and related xanthines in tea plants

For details see the Discussion section.

pathway (one directly from the nucleotide pool and the other from the breakdown of nucleic acids) are operative and that their relative significance varies according to tissue and conditions.

The observations presented in this paper are also compatible with the following lines that are described in other investigations, because the nucleotides may also be produced as a result of nucleic acid breakdown during nucleic acid turnover (Fig. 2b).

(1) The addition of RNA to tea leaves or tea callus tissue enhances caffeine formation (Serenkov, 1962; Ogutuga & Northcote, 1970a). (2) The withering of tea leaves in tea manufacture results in an increase in caffeine content (Wood & Chanda, 1955), a new synthesis of 2'- and 3'-isomers of AMP, CMP, GMP and UMP (Takino *et al.*, 1972; Takino & Imagawa, 1973), and net loss of RNA (Bhattacharyya & Ghosh, 1968).

Scheme 2 summarizes a hypothesis that agrees with the above-mentioned observations. Thus caffeine may be synthesized from the methylated purine nucleotides in the nucleotide pool via 7-methylxanthine and theobromine. It has been demonstrated that the purine ring in caffeine is directly derived from the purine nucleotides in the nucleotide pool rather than in nucleic acids (Suzuki & Takahashi, 1976a). The results of incorporation of more radioactivity from [2-¹⁴C]glycine than from L-[Me-¹⁴C]methionine into 7-methylxanthine and theobromine (Table 1) also indicate that there is active synthesis of these precursors from purine nucleotides synthesized by the pathways of purine biosynthesis *de novo*. However, further details on the pathways leading to the formation of 7-methylxanthine from the nucleotides in the nucleotide pool still remain obscure. In contrast, the formation of theophylline, which is also found in tea leaves (Franzke *et al.*, 1968), is explicable as the result of the methylation of N-1 of adenine in tRNA, that is, by analogous pathways demonstrated by Ogutuga & Northcote (1970b) for caffeine biosynthesis, theophylline is synthesized from 1-methyladenylic acid via 1-methylxanthine. Theophylline may also be finally converted into caffeine by the same enzyme catalysing caffeine formation from theobromine, but its conversion rate must be low in tea leaves (Suzuki & Takahashi, 1975b). Thus, if this pathway is operative, it must be a minor route.

We are grateful to the Radioisotope Research Center, Kyoto University, Kyoto, Japan for ultracentrifugation facilities.

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