Further Investigation of the Biosynthesis of Caffeine in Tea Plants (*Camellia sinensis* L.)

METHYLATION OF TRANSFER RIBONUCLEIC ACID BY TEA LEAF EXTRACTS

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(Received 14 April 1976)

1. The tRNA methyltransferase activity *in vitro* of leaves, cotyledons and roots of 85-day-old tea seedlings was studied. 2. The activity of extracts prepared from tea leaves with Polyclar AT (insoluble polyvinylpyrrolidone) had optimum pH 7.7 and was greatly influenced by thiol compounds, but only slightly by metal ions and ammonium acetate. 3. The activities of extracts, expressed per mg of protein, were as follows: roots > leaves > cotyledons. The only methylated base isolated after incubation with these preparations was 1-methyladenine. 4. The results did not support the view of involvement of methylation of nucleic acids in caffeine biosynthesis in tea plants. In contrast, it is suggested that theophylline is synthesized from the specific methylated precursor in nucleic acids, namely 1-methyladenylic acid, via 1-methylxanthine.

The biological methylation of nucleic acids at the polynucleotide level, not only in micro-organisms and animals but also in the plant kingdom, has now been well established and characterized (Borek & Srinivasan, 1965, 1966; Cantoni, 1975). The enzymes involved in these reactions are strictly specific both for the individual nucleic acids and for the individual bases. Among these enzymes, tRNA methyltransferases of various organisms have been subjected to a large number of investigations because of the essential role that tRNA molecules and their modified subspecies play in protein synthesis and its regulation (Craddock, 1970), in differentiation (Baliga *et al.*, 1965; Pillinger & Borek, 1969; Turkington, 1969) and possibly in malignant transformation (Borek & Kerr, 1972). It has been specifically emphasized that tRNA methyltransferase activity is considerably increased in neoplastic tissues when compared with normal tissue activity.

However, there are no reports on the methylation of tRNA *in vitro* associated with caffeine biosynthesis in both tea and coffee plants, although the possibility that the specific methylated purine precursor of caffeine might be produced as a result of nucleic acid methylation has been demonstrated in tea callus tissue (Ogutuga & Northcote, 1970). In the preceding paper (Suzuki & Takahashi, 1976), we revealed that three major and three minor products were obtained after hydrolysis of nucleic acids

prepared from tea shoot tips incubated with L-[Me-¹⁴C]methionine. Among them, the major product was identified as 1-methyladenine, whereas other products were not identified. An aim of the present paper is therefore to clarify these unknown products by using tea leaf extracts. As reported in the preceding paper (Suzuki & Takahashi, 1976), it was also shown that the methylation of nucleic acids in tea shoot tips occurred mainly in tRNA. Thus a study was made of methylation of tRNA with yeast tRNA molecules as methyl acceptors. That tRNA methyltransferases of spinach leaves (Srinivasan & Borek, 1963) and of pea seedlings (Birnstiel *et al.*, 1963) can catalyse the methylation of tRNA from bacterial cells has been described. Results are discussed in relation to the possible pathways for biosynthesis of caffeine and related methylxanthines in tea plants.

Materials and Methods

Chemicals

Materials were obtained from the sources given by Suzuki & Takahashi (1976) with the addition of S-adenosyl-L-[Me-¹⁴C]methionine (55 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K., and baker's-yeast tRNA from Boehringer, Mannheim, Germany.

Plant material

The techniques with tea seedlings were as described by Suzuki (1973). Tea leaves, plucked from rapidly

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growing 80–90-day-old seedlings, were used except where stated otherwise.

Preparation of enzyme extracts

All procedures were carried out at 4°C. The leaves were cut into small pieces and immediately frozen at –20°C for 1 h. A 10 g sample of the frozen leaves was ground with 6 g of washed Polyclar AT, about 3 g of washed sea sand and 50–60 ml of 100 mM-potassium phosphate buffer (pH 7.3), containing 5 mM-2-mercaptoethanol, 5 mM-EDTA and 0.5% sodium ascorbate, in a pre-chilled mortar. The homogenate was squeezed through four layers of cheesecloth and centrifuged for 20 min at 10000g. The supernatant solution was adjusted to 20% (w/v) saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$. The solution was stirred for 20 min, and the precipitated protein was removed by centrifugation for 15 min at 10000g and discarded. The supernatant was then adjusted to 60% (w/v) saturation by further additions of solid $(\text{NH}_4)_2\text{SO}_4$ and stirred for 20 min; the precipitate was collected by centrifugation (15 min; 10000g) and dissolved in the phosphate buffer used above. This protein solution was then applied to a column (30 cm × 2 cm) of Sephadex G-25. The column was eluted with 10 mM-potassium phosphate buffer (pH 7.3) containing 10 mM-2-mercaptoethanol and 2 mM-EDTA. The active effluent was collected and used as the enzyme source. The active effluent could be stored frozen at –20°C for at least 2 weeks without significant loss of activity.

Assay of tRNA methyltransferase activity

tRNA methyltransferase activity was measured by determination of the incorporation of labelled methyl groups from *S*-adenosyl-L-[$\text{Me-}^{14}\text{C}$]methionine into yeast tRNA. The assay medium contained, in a total volume of 250 μl : 25 μmol of phosphate buffer (pH 7.7), 250 μg of brewer's-yeast tRNA, 5 μmol of dithiothreitol, 0.05 μmol of MgCl_2 , 0.02 μCi (about 30000 c.p.m.) of *S*-adenosyl-L-[$\text{Me-}^{14}\text{C}$]methionine (55 mCi/mmol) and 120 μl of enzyme preparation (1.5–1.6 mg of protein). After 45 min incubation of the assay mixture at 37°C, the reaction was stopped by the addition of 2 ml of 5% (w/v) trichloroacetic acid. The tRNA precipitate was collected by centrifugation at 1000g for 10 min and the precipitate was washed with 5% (w/v) trichloroacetic acid followed by ethanol/diethyl ether (1:1, v/v) before being transferred to a Whatman glass-fibre filter (GF/C). The filters were washed with 10 ml of 95% (v/v) ethanol and 10 ml of diethyl ether and dried under an i.r. lamp. The filters were then placed in vials containing 5 ml of a toluene-based scintillator solution as described by Suzuki & Takahashi (1976). Measurements were made in a Beckman LS-100 liquid-scintillation counter. Controls to which no tRNA was added were incubated under the same

conditions. The results were corrected for the blank values thus obtained, and for the quenching by the precipitates.

Analysis of methylated products

The reaction mixture was the same as above, with the total volume increased to 2.5 ml. After incubation at 37°C for 120 min, an equal volume of phenol (saturated with water) was added to the incubation mixture. The phenol mixture was stirred at 4°C for 30 min and then centrifuged at 1000g for 20 min at 4°C. The upper aqueous phase was removed and the phenol phase (plus interphase) was washed with an equal volume of water. The aqueous phase and the wash were combined and dialysed against water to remove phenol and *S*-adenosyl-L-[$\text{Me-}^{14}\text{C}$]methionine. The tRNA was precipitated by the addition of 0.1 vol. of 20% (w/v) potassium acetate and 2 vol. of 95% (v/v) ethanol. The tRNA precipitate was collected by centrifugation at 1000g for 20 min at 4°C and the precipitate was washed with 50, 70 and 96% (v/v) ethanol and finally with diethyl ether. Hydrolysis of the dried tRNA and chromatographic analysis of methylated bases were as described by Suzuki & Takahashi (1976).

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), after precipitation with 5% (w/v) trichloroacetic acid, with bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Results

Table 1 shows that a cell-free extract of tea leaves can transfer ^{14}C -labelled methyl groups from *S*-adenosyl-L-[$\text{Me-}^{14}\text{C}$]methionine both to brewer's-yeast tRNA and to baker's-yeast tRNA; the former is more effective as a methyl acceptor than the latter. The enzyme activity was greatly affected by the addition of 5 μmol of dithiothreitol to the incubation mixture *in vitro*, but only slightly by 0.05 μmol of MgCl_2 (Table 1). At this concentration, only partial inhibition was given by K^+ , Mn^{2+} , Ca^{2+} , Co^{2+} and Fe^{2+} . Also no concentration of ammonium acetate had any significant effect on the enzyme activity. There was a slight incorporation of radioactivity in the incubation mixture without the acceptor, owing to the incorporation of methyl groups into protein.

Under the assay conditions described in the Materials and Methods section, the rate of methylation of tRNA by tea leaf extracts was linear with time up to at least 50 min and was proportional to the amount of extract added (up to at least 2.5 mg of protein/assay). The only base isolated after incubation of either brewer's-yeast tRNA or baker's-yeast

Table 1. Incorporation of *S*-adenosyl-L-[Me-¹⁴C]methionine into yeast tRNA by tea leaf extracts

The complete system contained, in a total volume of 250 μ l: 25 μ mol of (pH 7.7), 250 μ g of brewer's-yeast tRNA, 5 μ mol of dithiothreitol, 0.05 μ mol of MgCl₂, 0.02 μ Ci (about 30000 c.p.m.) of *S*-adenosyl-L-[Me-¹⁴C]methionine, and 120 μ l of extract from tea leaves (1.5–1.6 mg of protein; tea leaf extracts used as the enzyme source contained 10 mM-mercaptoethanol). Incubation was for 45 min at 37°C. Results are expressed as c.p.m./assay.

System	Methylation rate (c.p.m.)	Relative rate (%)
Complete (brewer's-yeast tRNA)	1750	100
Boiled enzyme	100	6
–Brewer's-yeast tRNA	200	11
–Dithiothreitol	490	28
–MgCl ₂	1710	98
–Dithiothreitol, MgCl ₂	480	27
Substituted baker's-yeast tRNA for brewer's-yeast tRNA	1510	86
Substituted L-[Me- ¹⁴ C]methionine and ATP for <i>S</i> -adenosyl-L-[Me- ¹⁴ C]methionine	50	3

Table 2. Effect of thiol compounds on the enzyme activity

The incubation mixture was as in Table 1 except that the thiol content was varied and the tea leaf extracts used as the enzyme source in assays 1 and 2 contained 10 mM- and 2.5 mM-mercaptoethanol respectively. Incubation was for 45 min at 37°C.

Assay	Reagents	Concn. (mM)	¹⁴ C incorporated (c.p.m./assay)
Assay 1	None	—	480
	Dithiothreitol	0.4	880
		2	1270
		4	1380
		8	1430
		20	1570
Assay 2		40	1370
	None	—	100
	Dithiothreitol	20	1660
	Mercaptoethanol	20	1260
	Cysteine	20	110

Table 3. tRNA methyltransferase activity of 85-day-old tea seedlings

Extracts were prepared from 10, 5 and 20 g fresh wt. of tea leaves, roots and cotyledons respectively. Incubation conditions were identical with those in Table 1.

Tissue	¹⁴ C incorporated (c.p.m./mg of protein) into	
	Brewer's-yeast tRNA	Baker's-yeast tRNA
Leaves	1020	920
Roots	3860	2960
Cotyledons	400	280

Fig. 1. Effect of pH and buffer composition on the enzyme activity

The incubation mixture contained 25 μ mol of either potassium phosphate buffer (●) or Tris/HCl buffer (○), at the indicated pH value. Otherwise the reaction mixture and the experimental conditions were as in Table 1.

tRNA with tea leaf extracts was 1-methyladenine, although at least three main peaks of radioactivity appeared after hydrolysis of nucleic acid preparations methylated *in vivo* in tea shoot tips.

Fig. 1 shows that 0.1 M-potassium phosphate buffer gave a greater activity than 0.1 M-Tris/HCl buffer, the former showing a pH optimum of 7.7 and the latter of 8.0.

Table 2 shows the action of reducing agents on the enzyme activity; the optimum stimulation occurred at 20 mM-dithiothreitol. 2-Mercaptoethanol could replace dithiothreitol, but cysteine could not.

Table 3 shows the distribution of tRNA methyltransferase activity in 85-day-old tea seedlings. Activity was detected in all parts of the plant: the activity expressed per mg of protein was highest for the extract of roots, next that of leaves, and that of cotyledons was lowest. In all cases the only methylated base isolated after incubation with these preparations was 1-methyladenine.

Discussion

The tRNA methyltransferase of 85-day-old tea seedlings described in the present paper is the enzyme specifically methylating *N*-1 of adenine, i.e. adenine 1-methyltransferase. Judging from the preparation procedures and assay conditions for this enzyme, its basic features appear to be similar to those of tRNA methyltransferase from other cells, although there may be differences in detail (for example, see the report of Pegg & Hawks, 1974).

The reasons for failure to detect other methyltransferases, when the enzyme extracts were incubated with yeast tRNA as a methyl acceptor, may be extraction procedures rather than assay conditions used in the present experiments. These procedures are based on the report of Sanderson (1966). Birnstiel *et al.* (1963) reported three tRNA methyltransferases, i.e. uracil 5-methyltransferase, guanine 1-methyltransferase and adenine *N*⁶-methyltransferase, of nucleoli from 36h-old pea seedlings. Hence these methyltransferase activities appear to be closely associated with nuclei, especially with nucleoli. A modification of extraction procedures, e.g. the use of a soluble polyvinylpyrrolidone in place of an insoluble one (Loomis, 1969), should permit isolation of nucleolar preparations from tea leaves for detection of other methyltransferase activities.

Thus unexpectedly, we were unable to identify the unknown products described in the preceding paper (Suzuki & Takahashi, 1976), but the results of the present experiments are satisfactory in disproving the view that nucleic acid methylation is involved in caffeine biosynthesis (Ogutuga & Northcote, 1970), because 1-methyladenine was obtained as the major product after hydrolysis of nucleic acids methylated in tea shoot tips (Suzuki & Takahashi, 1976). 7-Methylguanine was not obtained after this hydrolysis. The results (Table 3) also show that the adenine 1-methyltransferase activity of root preparations is much higher than that of leaf preparations, although the activity of the theobromine- and caffeine-synthesizing enzymes was only detected in leaf preparations (T. Suzuki & E. Takahashi, unpublished work). Our hypothesis for

caffeine biosynthesis depends on an assumption of the methylation of the nucleotides in the nucleotide pool; hence it is necessary at the moment to keep an open mind about the major precursor of caffeine, i.e. the origin of the purine ring in caffeine, until these methylating systems are demonstrated *in vitro*. In contrast, assuming that, by pathways analogous to those demonstrated for caffeine biosynthesis by Ogutuga & Northcote (1970), theophylline is synthesized from nucleic acids, it is possible that theophylline is produced from 1-methyladenylic acid, formed as a result of nucleic acid methylation and subsequent degradation, via 1-methylxanthine (Suzuki & Takahashi, 1975).

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