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Biosynthesis of Caffeine by Tea-Leaf Extracts

ENZYMIC FORMATION OF THEOBROMINE FROM 7-METHYLXANTHINE AND OF CAFFEINE FROM THEOBROMINE

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1. Extracts prepared from tea leaves with Polyclar AT (insoluble polyvinylpyrrolidine) contained two methyltransferase activities catalysing the transfer of methyl groups from *S*-adenosylmethionine to 7-methylxanthine, producing theobromine, and to theobromine, producing caffeine. 2. The methyltransferases exhibited the same pH optimum (8.4) and a similar pattern of effects by metal ions, thiol inhibitors and metal-chelating reagents, both for theobromine and caffeine synthesis. Mg^{2+}, Mn^{2+} and Ca^{2+} slightly stimulated enzyme activity but they were not essential. Paraxanthine was shown to be most active among methylxanthines, as the methyl acceptor. However, the formation of paraxanthine from 1-methylxanthine was very low and that from 7-methylxanthine was nil, suggesting that the synthesis of caffeine from paraxanthine is of little importance in intact plants. Xanthine, xanthosine, XMP and hypoxanthine were all inactive as methyl acceptors, whereas [2-^{14}C]xanthine and [8-^{14}C]hypoxanthine were catabolized to allantoin and urea by tea-leaf extracts. The apparent K_m values are as follows: 7-methylxanthine, 1.0×10^{-3}M; theobromine, 1.0×10^{-3}M; paraxanthine, 0.2×10^{-3}M; *S*-adenosylmethionine, 0.25×10^{-4}M (with each of the three substrates). 3. The results suggest that the pathway for caffeine biosynthesis is as follows: 7-methylxanthine → theobromine → caffeine. In contrast, it is suggested that theophylline is synthesized from 1-methylxanthine. The methyl groups of the purine ring of caffeine are all derived directly from the methyl group of *S*-adenosylmethionine. Little is known about the pathways leading to the formation of 7-methylxanthine. 4. A good correlation between caffeine synthesis and shoot formation or growth of tea seedlings was shown, suggesting that the methylating systems in caffeine synthesis are closely associated with purine nucleotide and nucleic acid metabolism in tea plants.

Much work on caffeine biosynthesis using either intact plants (Wanner & Blaim, 1961; Anderson & Gibbs, 1962; Inoue & Adachi, 1962; Preusser & Serenkov, 1963; Preusser, 1967; Inoue, 1971; Konishi *et al.*, 1972*a*,*b*; Suzuki, 1972, 1973; Keller *et al.*, 1972; Konishi & Oishi, 1973) or callus tissue (Ogutuga & Northcote, 1970*a*,*b*; Keller *et al.*, 1972) has been carried out. These studies have established that caffeine is synthesized from the same precursors utilized for purine and methyl group synthesis in other systems. Theobromine appears to be the immediate precursor of caffeine (Ogutuga & Northcote, 1970*a*; Suzuki, 1973; Suzuki & Takahashi, 1974). *S*-Adenosylmethionine seems to be the actual methyl donor in the methylations of caffeine precursors (Suzuki, 1972, 1973).

Much less is known about the pathways leading to the formation of theobromine, or about the methylating systems involved in caffeine biosynthesis. There have been certain difficulties in approaching these questions owing to the limitations inherent in studies with intact plants. Callus-tissue culture may be advantageous for certain aspects of the study of caffeine biosynthesis. Ogutuga & Northcote (1970*a*,*b*) used tea callus tissue and obtained good results, which compare favourably with our results using detached parts of tea plants (Suzuki, 1973; Suzuki & Takahashi, 1974).

In contrast with these studies with intact plants or callus tissue, cell-free systems are more advantageous, because the direct proofs for the involvement of *S*-adenosylmethionine and the order of methylations of the purine ring of caffeine can be obtained only by the use of cell-free systems. One of the difficulties in extracting active enzymes from tissues containing high concentrations of phenolic compounds, e.g. tea, coffee and peppermint leaves, arises from the denaturation of proteins by these compounds. However, techniques have been developed that enable soluble solutions to be extracted from such tissues (Loomis, 1969), and these techniques have not been applied to extraction of the enzymes involved in

caffeine biosynthesis from tea and coffee leaves. The enzymes reported in this paper are methyltransferases from tea leaves, which catalyse the transfer of methyl groups from *S*-adenosylmethionine to 7-methylxanthine, producing theobromine, and to theobromine, producing caffeine. Attempts to extract similar enzymes from coffee leaves were unsuccessful. These results suggest that caffeine is synthesized from 7-methylxanthine via theobromine, and that xanthine, xanthosine, XMP and hypoxanthine cannot serve as the direct precursors of 7-methylxanthine.

Materials and Methods

Chemicals

Materials were obtained from the following sources: 3-methylxanthine, courtesy of Dr. M. Sekiya, Shizuoka College of Pharmacy, Shizuoka, Japan; 1-methylxanthine, courtesy of Dr. S. Schwimmer, Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, Calif., U.S.A.; paraxanthine (1,7-dimethylxanthine), courtesy of Dr. G. B. Elion, Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, N.C., U.S.A.; 7-methylxanthine, prepared from XMP as described by Jones & Robins (1963); xanthine, xanthosine, hypoxanthine, theobromine, theophylline and caffeine from Nakarai Chemical Co., Kyoto, Japan; XMP, ATP, crystallized bovine serum albumin and *S*-adenosyl-L-methionine from Sigma Chemical Co., St. Louis, Mo., U.S.A.; *S*-adenosyl-L-[*Me*-^{14}C]methionine (55mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.; L-[*Me*-^{14}C]methionine (53mCi/mmol), [2-^{14}C]xanthine (48mCi/mmol) and [8-^{14}C]hypoxanthine (47mCi/mmol) from Le Commissariat à l'Énergie Atomique, Paris, France; Polyclar AT (insoluble polyvinylpyrrolidine) from General Aniline and Film Corp., Dyestuff and Chemical Division, New York, N.Y., U.S.A.

Plant material

The methods for growth of tea seedlings were as described by Suzuki (1973). Tea leaves, plucked from 75–85-day-old seedlings that were growing rapidly, were used except as otherwise stated.

The coffee leaves used for this study were harvested from plants growing rapidly at 25°C in normal daylight in a greenhouse of the Nippon Shinyaku Institute for Botanical Research, Kyoto, Japan.

Methods

Preparation of extracts. All procedures were carried out at 4°C. The leaves were plucked from 75–85-day-old tea seedlings, cut into small pieces and immediately frozen at −20°C for 1h. A 5g sample of the frozen leaves was ground with 3g of washed Polyclar AT (which is necessary to obtain the extract exhibiting enzyme activity), about 3g of washed sea sand and 30–40ml of 100mM-potassium phosphate buffer (pH7.3) containing 5mM-2-mercaptoethanol, 5mM-EDTA and 0.5% sodium ascorbate in a pre-chilled mortar. The homogenate was squeezed through four layers of cheesecloth and centrifuged for 20min at 10000g. The supernatant solution (referred to as the crude extract) was brought to 20% (w/v) saturation by addition of solid $(NH_4)_2SO_4$. The solution was stirred for 20min, and the precipitated protein was removed by centrifugation for 15min at 10000*g* and discarded. The supernatant was then adjusted to 60% (w/v) saturation by further additions of solid $(NH_4)_2SO_4$ and stirred for 20min; the precipitate was collected by centrifugation (15min; 10000*g*) and dissolved in the phosphate buffer used above. This protein solution was then applied to a column (30cm×2cm) of Sephadex G-25. The column was eluted with 10mM-potassium phosphate buffer (pH7.3) containing 2mM-2-mercaptoethanol and 2mM-EDTA. The active effluent was collected and used as the enzyme source. The $(NH_4)_2SO_4$ fractionation and Sephadex G-25 filtration are necessary to remove endogenous substrate and product, since the crude extract contains significant amounts of caffeine and smaller amounts of related xanthines.

Assay of enzyme activities. Enzyme activity was assayed immediately after Sephadex G-25 filtration, since more than 50% of the activity was lost within 12h at 4°C. The storage of the extract with the additions of 10mM-2-mercaptoethanol and 12.5% (w/v) glucose at −20°C did not prevent the loss of enzyme activity.

The assay is based on the transfer of ^{14}C-labelled methyl groups from *S*-adenosyl-L-[*Me*-^{14}C]methionine to 7-methylxanthine, forming theobromine, or to theobromine, forming caffeine. The standard assay mixture contained 20μl each of 500mM-Tris–HCl buffer (pH8.4), 1mM-7-methylxanthine or other substrates, 10μl each of 2mM-$MgCl_2$, 0.2μCi (about 24000c.p.m.) of *S*-adenosyl-L-[*Me*-^{14}C]-methionine (55μCi/μmol) and 40μl of enzyme preparation (80–100μg of protein). After 10min incubation of the assay mixture at 30°C, the reaction was stopped by the addition of 50μl of 1.5M-$HClO_4$. After centrifugation of the mixture (1000*g*; 15min), portions (50μl) of the supernatant were subjected to ascending chromatography on Whatman 3MM paper in solvent (1) butan-1-ol–acetic acid–water (4:1:1, by vol.). Theobromine or other related methylxanthines were co-chromatographed on each spot as authentic materials and detected by u.v. quenching (253.7nm). The chromatogram was developed and the positions of the u.v.-absorbing spots corresponding to the authentic materials were marked in pencil,

then cut from the papers and transferred to vials containing 10ml of a toluene-based scintillator solution. This solution contained 4g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre of toluene. Measurements were made in a Horiba LS-500 liquid-scintillation counter. Measurements were corrected for background radioactivity.

Identification of the reaction products. For characterization of the reaction products, the enzyme reaction was carried out with a larger incubation volume of 1ml at 30°C for 1h. The reaction was stopped by the addition of 0.3ml of 1.5M-$HClO_4$. After centrifugation of the mixture (1000*g*; 15min), the supernatant was applied in increments to Whatman 3MM paper in a band 40cm long. Ascending paper chromatography in solvent (1) was then carried out overnight. The chromatograms were dried and radioactive areas were detected as follows. Strips (3cm×1cm) were cut down the direction of run of each sample, such that the long axis of each strip was at right-angles to the direction of chromatographic movement of the sample. The strips were then transferred to vials containing 5ml of the toluene-based scintillator solution, and their radioactivities were measured, as described above.

The bands corresponding to the radioactivity peak on the chromatograms were then excised with sufficient lateral margins to permit vertical insertion of the strips into a trough for descending elution with water; a point is cut at the lower end. The radioactive materials were completely eluted by 10–12ml of water. The effluents were then concentrated to a small volume in a rotary evaporator under vacuum and rechromatographed on Whatman no. 1 paper in solvent systems (1), (2) ethanol–acetic acid–water (81:5:14, by vol.) and (3) pyridine–conc. NH_3 (sp.gr. 0.90) (47:3, v/v), or on silica-gel plates (Eastman Chromatogram Sheets 6060) in solvent system (4) chloroform–carbon tetrachloride–methanol (8:5:1, by vol.). Appropriate theobromine and methylxanthine markers were co-chromatographed on each chromatogram as authentic materials. The positions of the u.v.-absorbing spots on the papers were marked in pencil and the radioactive areas were detected as described above. The radioactive areas on the silica-gel plate were detected by radioautography with Fuji X-ray film. The authentic materials on the silica-gel plate were detected by u.v. quenching (253.7nm).

Determination of protein. This was determined by the method of Lowry *et al.* (1951), after precipitation with 5% (w/v) trichloroacetic acid, with bovine serum albumin as standard.

Determination of caffeine. The methods for extracting the caffeine and the spectrophotometric method for its determination were as described by Konishi *et al.* (1972*b*).

Determination of dry weight. The dry weight of tea shoots was measured after each sample of fresh tissue had been cut into small pieces, dried at 80°C for 2h and placed in a vacuum desiccator overnight.

Results

Synthesis of theobromine from 7-methylxanthine and of caffeine from theobromine by tea-leaf extracts

Theobromine appears to be the immediate precursor of caffeine (Ogutuga & Northcote, 1970*a*; Suzuki, 1973; Suzuki & Takahashi, 1974). Thus at first we studied enzymic synthesis of theobromine from 7-methylxanthine or 3-methylxanthine, and of caffeine from theobromine. Extracts (Sephadex G-25 fraction) from tea leaves were incubated in the presence of *S*-adenosyl-L-[*Me*-^{14}C]methionine as the methyl donor and 7-methylxanthine (or other substrate) as the methyl acceptor (Table 1). After incubation for 1h at 30°C, the radioactive materials produced by tea-leaf extracts were separated by paper chromatography on Whatman 3MM paper in solvent (1) and rechromatographed on Whatman no. 1 paper or on silica-gel plate in each of the systems described in the Materials and Methods section. Table 1 shows the summary of these experiments. The products formed from each of the three compounds: 7-methylxanthine, 3-methylxanthine and theobromine by leaf extracts were identified as theobromine, theophylline and caffeine, respectively.

The requirements for enzymic syntheses of theobromine and caffeine are also given in Table 1, showing that no methylated product was formed in the reaction systems without either the acceptor, or the enzyme. Mg^{2+} stimulated the enzyme activity slightly, although it was not apparently essential. There was also no reaction when L-[*Me*-^{14}C]methionine, ATP and Mg^{2+} were replaced for *S*-adenosyl-L-[*Me*-^{14}C]-methionine as the methyl donor (Table 1).

It was also shown that no reaction was observed when the crude extracts were prepared without Polyclar AT. Although no detailed studies were made on the effect of the amount of Polyclar AT on enzyme extraction from tea leaves, the amount reported by Sanderson (1966) seemed to give good results.

Properties of tea-leaf methyltransferase catalysing caffeine synthesis

Effect of time and enzyme concentration. The rate of formation of theobromine and caffeine by tea-leaf extracts was shown to be linear with time up to at least 7min and was proportional to the amount of extract added (up to at least 60μg of protein/assay) under the conditions of the standard assay described in the Materials and Methods section. There was no

Table 1. *Incorporation of S-adenosyl-L-[Me-^{14}C]methionine into methylxanthines by tea-leaf extracts*

The complete system contained 20 μl each of 500 mM-Tris–HCl buffer (pH 8.4), 1 mM-7-methylxanthine (or other substrates), 10 μl (0.02 μCi, about 24000 c.p.m.) of *S*-adenosyl-L-[*Me*-^{14}C]methionine (55 mCi/mmol), 10 μl of 2 mM-$MgCl_2$, and 40 μl of extract from tea leaves (80–100 μg of protein). L-[*Me*-^{14}C]Methionine and ATP were substituted for *S*-adenosyl-L-[*Me*-^{14}C]-methionine as the methyl donor. The incubation was done for 10 min at 30°C. The identification of the reaction products, with a larger incubation volume of 1 ml, was performed as described in the Materials and Methods section. Results are expressed as c.p.m./assay. n.d., Not detectable.

System	^{14}C incorporated into ...	7-Methylxanthine		3-Methylxanthine		Theobromine
		Theobromine	Paraxanthine	Theobromine	Theophylline	Caffeine
Complete		6415	n.d.	n.d.	30	1830
Minus 2 mM-$MgCl_2$		5830	n.d.	n.d.	20	1750
Minus acceptor		n.d.	n.d.	n.d.	n.d.	n.d.
Minus enzyme		n.d.	n.d.	n.d.	n.d.	n.d.
Substituted L-[*Me*-^{14}C]methionine and ATP for *S*-adenosyl-L-[*Me*-^{14}C]-methionine		n.d.	n.d.	n.d.	n.d.	n.d.

reaction when the enzyme preparations were heated in a boiling-water bath for 5 min before the assay.

Stability of methyltransferase activity. The enzyme in Sephadex G-25 column effluents lost its activity usually by an average of 4%/h over the first 12 h at 4°C after elution. The addition of 10 mM-2-mercaptoethanol and 12.5% (w/v) glucose to the effluents and storage at −20°C only slightly prevented the loss of enzyme activity; even in this case, more than 50% of enzyme activity was lost within 1 day. No further experiments were made on the stabilization of methyltransferase activity.

pH profile of methyltransferase activity. The pH optimum in 0.5 M-Tris–HCl buffer at 30°C, with 1 mM-7-methylxanthine as substrate, appeared sharply at pH 8.4 (Fig. 1). In contrast with this, the pH optimum for caffeine synthesis from theobromine was broadly around pH 8.4. In 0.5 M-potassium phosphate buffer, the maximum rate of syntheses of both products from 7-methylxanthine and from theobromine was at pH 8.0.

Effect of metal ions on methyltransferase activity. The enzyme did not require bivalent cations. Mg^{2+}, Ca^{2+} and Mn^{2+} only slightly stimulated both theobromine and caffeine formation. The optimum concentration of Mg^{2+} on the enzyme activity was 2 mM. Cu^{2+} and Hg^{2+} were strongly inhibitory at 1 mM, whereas Co^{2+}, Zn^{2+}, Cd^{2+} and Fe^{2+} exerted only a partial inhibition at this concentration.

Effect of inhibitors on methyltransferase activity. Table 2 illustrates the action of inhibitors on the enzyme activity. There was no significant inhibition by thiol reagents such as 100 mM-iodoacetate and 0.5 mM-*p*-chloromercuribenzoate; the reaction was even stimulated with 1 mM-iodoacetate (final concn.) in the reaction mixture.

All chelating agents tested also only partially inhibited both theobromine and caffeine formation. The absence of any essential requirement for metal ions was therefore consistent with the results of inhibition experiments by chelating agents.

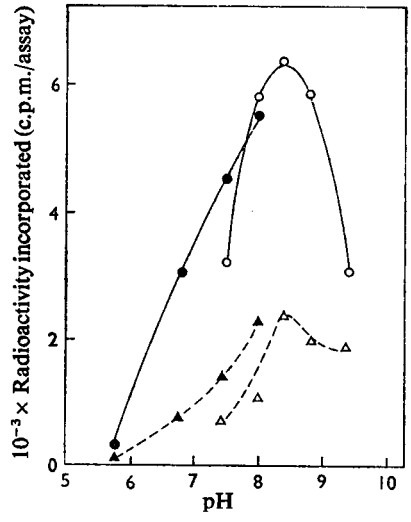


Fig. 1. *Effect of pH on methyltransferase activity*

The incubation mixture contained 20 μl each of 500 mM-Tris–HCl buffer (○, △) or 500 mM-potassium phosphate buffer (●, ▲), at the indicated pH value, and 1 mM-7-methylxanthine (●, ○) or 1 mM-theobromine (▲, △), 10 μl (0.02 μCi, about 24000 c.p.m.) of *S*-adenosyl-L-[*Me*-^{14}C]methionine (55 mCi/mmol), 10 μl of 2 mM-$MgCl_2$, and 40 μl of extract from tea leaves (80–100 μg of protein). The incubation was for 10 min at 30°C.

Substrate specificity of methyltransferase. To investigate the pathways for caffeine biosynthesis *in vitro*, xanthine, xanthosine, XMP, hypoxanthine and various methylxanthines were tested as acceptors (Table 3). The pH optimum for methylation of methylxanthines was in the range pH 8.0–9.0 in 0.5 M-Tris–

Table 2. *Effect of inhibitors on the enzyme activity*

Tea-leaf extract (40 μl; 80–100 μg of protein) was preincubated for 5 min with 10 μl of inhibitors at the concentrations indicated in 20 μl of 500 mM-Tris–HCl buffer (pH 8.4) at 30°C and then the activity was measured under standard assay conditions.

	7-Methylxanthine		Theobromine	
Reagents (mM)	Methylation rate (c.p.m.)	Relative rate (%)	Methylation rate (c.p.m.)	Relative rate (%)
None	7490	100	980	100
Thiourea (5)	7090	94	960	98
α,α'-Dipyridyl (5)	6100	81	810	83
EDTA (5)	6230	83	1010	101
Monoiodoacetate (100)	6630	89	950	97
Monoiodoacetate (10)	7780	104	1010	101
p-Chloromercuribenzoate (0.5)	5170	69	850	87
KCN (1)	7920	105	1150	117
NaN_3 (10)	7380	99	1040	106

Table 3. *Substrate specificity of methyltransferase*

The incubation mixture contained 20 μl of 500 mM-Tris–HCl buffer (pH 8.4), 20 μl of the substrate (methyl acceptor) at the concentrations listed, 10 μl (0.02 μCi, about 24000 c.p.m.) of *S*-adenosyl-L-[*Me*-^{14}C]methionine (55 mCi/mmol), 10 μl of 2 mM-$MgCl_2$, and 40 μl of extract from tea leaves. The incubation was for 60 min at 30°C. The identification of the reaction products, with a larger incubation volume of 1 ml, was performed as described in the Materials and Methods section.

Substrate (mM)		Methylated product	Relative methylation (%)
7-Methylxanthine	(1)	Theobromine	100
3-Methylxanthine	(1)	Theophylline	<1
1-Methylxanthine	(1)	Theophylline	4–5
		Paraxanthine	3–4
Theobromine	(1)	Caffeine	20–30
Theophylline	(1)	Caffeine	2–3
Paraxanthine	(1)	Caffeine	200–300
Xanthine	(0.5)	—	0
Xanthosine	(1)	—	0
XMP	(1)	—	0
Hypoxanthine	(0.5)	—	0

HCl buffer and at pH 8.0 in 0.5 M-potassium phosphate buffer. 3-Methylxanthine was almost inactive throughout the range pH 5.8–9.4. 1-Methylxanthine produced two products, paraxanthine and theophylline, whereas two other monomethylxanthines (7-methylxanthine and 3-methylxanthine) produced only one product, theobromine or theophylline respectively.

Unexpectedly, paraxanthine was the most active among methylxanthines; the formation of caffeine from paraxanthine was 2 or 3 times higher than that of theobromine from 7-methylxanthine. However, the formation of paraxanthine from 1-methylxanthine was very low and that from 7-methylxanthine was zero, indicating that paraxanthine is not the normal precursor of caffeine *in vivo*. In contrast, the synthesis of caffeine from theophylline and of theophylline from 1-methylxanthine or 3-methylxanthine was very low, indicating that the synthesis of caffeine via theophylline is scarcely the main pathway *in vivo*.

Xanthine, xanthosine, XMP and hypoxanthine were totally inactive (Table 3), suggesting that these compounds cannot serve as the direct or indirect precursors of 7-methylxanthine. However, it is still possible that these compounds can act as methyl acceptors under different conditions, because the conditions tested are the best for methylation of methylxanthines and these optimal conditions may not be suitable for other methylation reactions.

The extracts were unable to methylate xanthine and hypoxanthine when [2-^{14}C]xanthine or [8-^{14}C]-hypoxanthine, as a methyl acceptor, and *S*-adenosylmethionine, as the methyl donor, were incubated with tea-leaf extracts in 0.5 M-Tris–HCl buffer (pH 8.4) at 30°C for 60 min. The products formed from [2-^{14}C]-xanthine were identified as allantoin and urea by paper chromatography in solvents (1) and (2). Similarly, xanthine and allantoin were obtained by incubation with [8-^{14}C]hypoxanthine. In neither case was incorporation of the label into methylxanthines observed.

Effect of substrate concentration on methyltransferase activity. The rate of production of theobromine and caffeine was measured at various concentrations of 7-methylxanthine, theobromine, paraxanthine and *S*-adenosylmethionine by varying the standard assay mixture as follows: 20 μl each of 500 mM-Tris–HCl buffer (pH 8.4), *S*-adenosyl-L-[*Me*-^{14}C]methionine (55 μCi/μmol), enzyme preparation and 40 μl of 7-methylxanthine (or other substrates). The amount of radioactivity of *S*-adenosyl-L-[*Me*-^{14}C]methionine

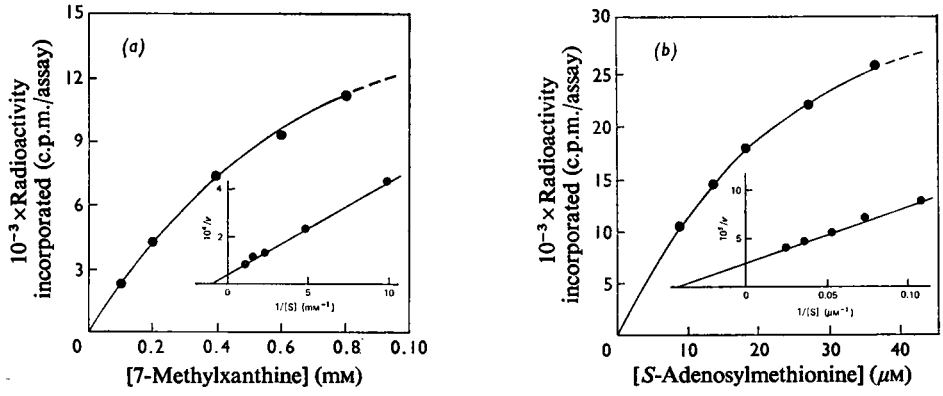


Fig. 2. *Effect of concentration of (a) 7-methylxanthine and (b) S-adenosylmethionine on the enzyme activity*

Incubations were conducted as described in the Results section for 5 min at 30°C. The specific activity of the enzyme preparations was 1.8 nmol of theobromine/5 min per mg of protein. Velocities are radioactivity (c.p.m.) incorporated into theobromine/assay. The K_m values for 7-methylxanthine and *S*-adenosylmethionine, from the double-reciprocal plot in the insert, were 1.0×10^{-3} M and 0.25×10^{-4} M respectively.

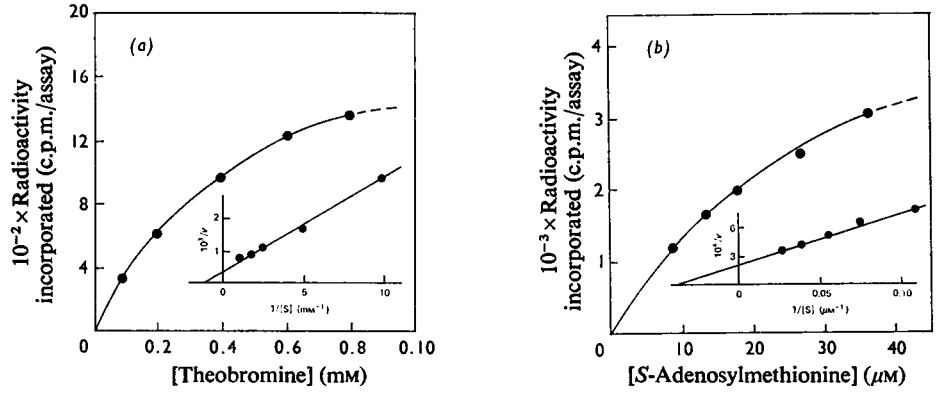


Fig. 3. *Effect of concentration of (a) theobromine and (b) S-adenosylmethionine on the enzyme activity*

Incubations were conducted as described in the Results section for 5 min at 30°C. The specific activity of the enzyme preparations was 0.3 nmol of caffeine/5 min per mg of protein. Velocities are radioactivity (c.p.m.) incorporated into caffeine/assay. The K_m values for theobromine and *S*-adenosylmethionine, from the double-reciprocal plot in the insert, were 1.0×10^{-3} M and 0.25×10^{-4} M respectively.

added was 0.04 μCi (48000 c.p.m.) when the incubation was done with 7-methylxanthine (or other substrates) at the indicated concentrations. The concentration of 7-methylxanthine (or other substrate) added for determination of K_m values for *S*-adenosylmethionine was 2 mM. These modifications were needed because the methyltransferase reaction was linear with time and enzyme concentration. It should be noted that these estimates are only approximate, because the K_m values given below may change on further purification of the enzyme preparations.

The rate of production of theobromine at various concentrations of 7-methylxanthine and *S*-adenosylmethionine is shown in Figs. 2(*a*) and 2(*b*). From the double-reciprocal plot in the insert, the calculated K_m values were 1.0×10^{-3} M and 0.25×10^{-4} M for 7-methylxanthine and *S*-adenosylmethionine respectively.

The rate of production of caffeine at various concentrations of theobromine and *S*-adenosylmethionine is shown in Figs. 3(*a*) and 3(*b*). From the double-reciprocal plot in the insert, the calculated K_m values were 1.0×10^{-3} M and 0.25×10^{-4} M for

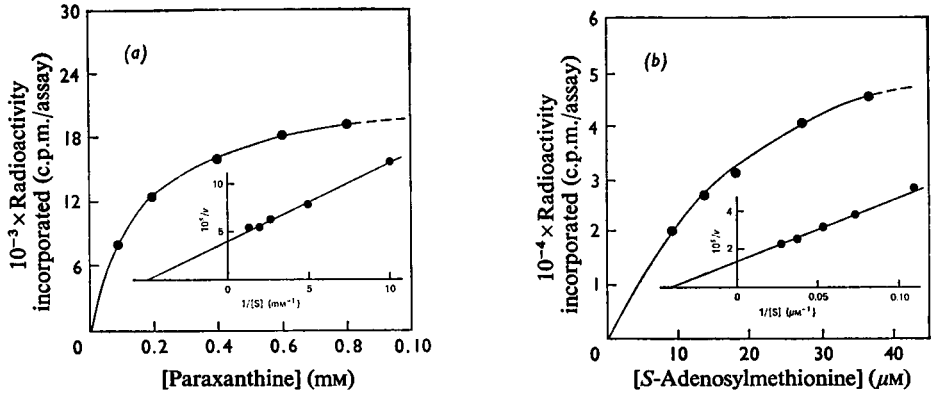


Fig. 4. *Effect of concentration of (a) paraxanthine and (b) S-adenosylmethionine on the enzyme activity*

Incubations were conducted as described in the Results section for 5 min at 30°C. The specific activity of the enzyme preparations was 4.4 nmol of caffeine/5 min per mg of protein. Velocities are radioactivity (c.p.m.) incorporated into caffeine/assay. The K_m values for paraxanthine and *S*-adenosylmethionine, from the double-reciprocal plot in the insert, were 0.2×10^{-3} M and 0.25×10^{-4} M respectively.

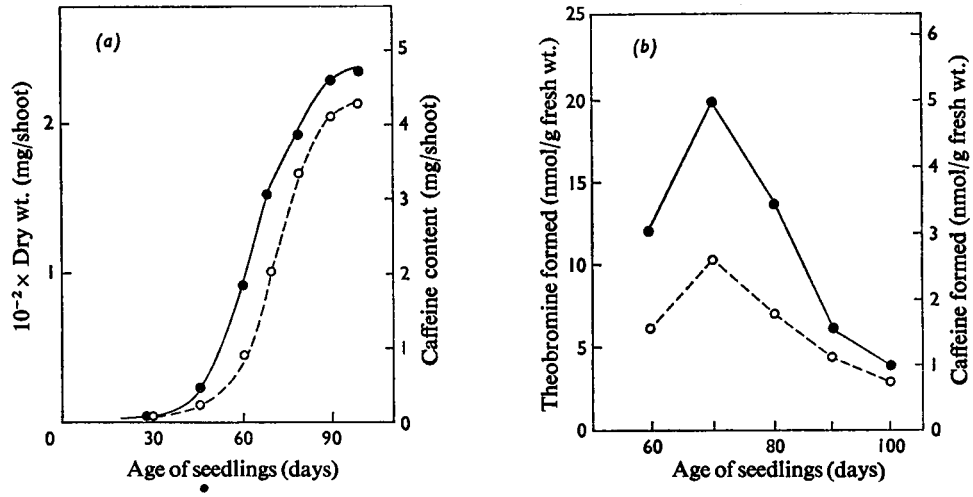


Fig. 5. *Changes in (a) dry weight and caffeine content and (b) methyltransferase activity in the shoots of tea seedlings during growth*

After sterilization by 0.004% methoxyethylmercuric chloride solution for 4 h, seeds of tea (1.0–1.5 g wt.) were germinated and grown from October to January, in pots of vermiculite in normal daylight in a greenhouse maintained at 25°C during the day and at 15°C at night. (*a*) Batches of five shoots of tea seedlings were used for each determination of the dry weight(●) and the caffeine content (○) of tea shoots. For further details see the text. (*b*) Theobromine synthesis from 7-methylxanthine (●) and caffeine synthesis from theobromine (○) by tea-leaf extracts. Extracts were prepared from 3 g of fresh wt. of tea leaves. Further details are given in the text.

theobromine and *S*-adenosylmethionine respectively.

The rate of production of caffeine at various concentrations of paraxanthine and *S*-adenosylmethionine is shown in Figs. 4(*a*) and 4(*b*). From the double-reciprocal plot in the insert, K_m values of 0.20×10^{-3} M and 0.25×10^{-4} M for paraxanthine and *S*-adenosylmethionine respectively were calculated.

Caffeine synthesis and methyltransferase activity in relation to growth

Tea seedlings were examined at various stages of growth for their content of caffeine and for activity of methyltransferase. The results (Figs. 5*a* and 5*b*) indicate a good correlation among the pattern of changes in shoot growth, caffeine content and enzyme activity. The weight of tea shoots and the content of caffeine increased continuously and reached a maximum value at 100 days, whereas considerable increase of dry weight of tea shoots and considerable synthesis of caffeine occurred in 60–80-day-old seedlings. The changes in enzyme activity of methyltransferase catalysing both theobromine and caffeine syntheses exhibited a similar pattern; the activity of methyltransferase reached its highest value at 70 days and then decreased.

Experiments with extracts from leaves of mature tea and coffee plants

Extracts prepared from rapidly growing shoot tips of mature tea plants exhibited methyltransferase activity, which has similar characteristics to those obtained from leaves of 75–85-day-old seedling plants.

Extracts prepared from coffee leaves, by the same methods as those used for the extraction of active enzymes from tea leaves, proved incapable of synthesis of methylxanthines from *S*-adenosyl-L-[*Me*-^{14}C]methionine. Even after addition of an equal weight or more of Polyclar AT to fresh coffee leaves, the crude extract from coffee leaves was shown to brown badly and no enzyme activity could be detected. No further attempts to extract active enzymes from coffee leaves, including seedling plants, were made.

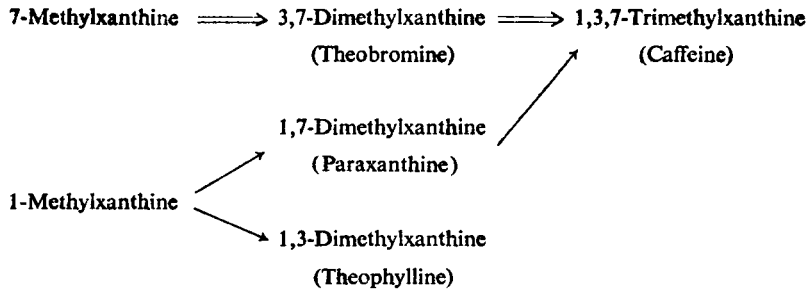
Discussion

Previous work on caffeine biosynthesis, in which detached parts of tea plants (Suzuki, 1972, 1973; Suzuki & Takahashi, 1974) or tea callus-tissue culture of tea plants (Ogutuga & Northcote, 1970*a*,*b*) were used, indicated that theobromine is the immediate precursor of caffeine and that *S*-adenosylmethionine serves as the actual methyl donor for caffeine biosynthesis. The present paper describes a further study of caffeine biosynthesis by using tea-leaf extracts; the results suggest that caffeine is synthesized from 7-methylxanthine via theobromine and that xanthine, xanthosine, XMP and hypoxanthine do not serve as the direct or indirect precursors of 7-methylxanthine under these conditions.

Table 1 clearly shows that *S*-adenosylmethionine is the actual methyl donor both for theobromine and caffeine syntheses. The failure of methionine to act as a methyl donor, even in the presence of ATP and $MgCl_2$, may be ascribed to the specific conditions used, since there is evidence for the formation of *S*-adenosylmethionine from methionine in tea plants (Suzuki, 1972). Although Suzuki & Takahashi (1974) could not show the precursor of 7-methylxanthine, which may be derived from the purine nucleotides synthesized via the pathways of purine nucleotides *de novo* or via the pathways of nucleic acid catabolism, *S*-adenosylmethionine appears to act as the actual methyl donor for the methylation of 7-methylxanthine precursor. Ogutuga & Northcote (1970*b*) demonstrated that 7-methylxanthine is derived from 7-methylguanylic acid in nucleic acid. If this hypothesis is right, *S*-adenosylmethionine is the actual methyl donor of 7-methylxanthine precursor, because nucleic acids are now well known to be methylated by *S*-adenosylmethionine in the high polymer levels (Borek & Srinivasan, 1965). Thus the methyl groups of caffeine are concluded to be all derived directly from the methyl group of *S*-adenosylmethionine. Further, it is suggested that all other C_1 compounds tested in the biosynthesis of caffeine in coffee plants (Anderson & Gibbs, 1962) are transferred to the methyl groups of caffeine via the C_1 pathways and directly from the methyl group of *S*-adenosylmethionine.

Surprisingly, paraxanthine is most active as the methyl acceptor among various methylxanthines tested (Table 3). However, the synthesis of caffeine via paraxanthine from 1-methylxanthine may be unlikely *in vivo*, because the rate of synthesis of paraxanthine from 1-methylxanthine is very low. Further, paraxanthine cannot be synthesized from 7-methylxanthine. Presumably therefore the synthesis of caffeine from 1-methylxanthine via paraxanthine is of little importance, although it would occur in leaves of intact plants. Paraxanthine is most active as an exogenous substrate. These observations are consistent with those made on caffeine biosynthesis by using the intact plants or callus tissue of tea plants (Ogutuga & Northcote, 1970*a*,*b*; Suzuki, 1973; Suzuki & Takahashi, 1974), and are further supported by the facts that theobromine and caffeine are normal constituents of the leaves both of intact and manufactured plants, i.e. green and black tea (Franzke *et al.*, 1968). In addition, from our present results (Table 3), the formation of theophylline, which is also found in tea leaves (Franzke *et al.*, 1968), is explained as the result of the methylation of 1-methylxanthine rather than that of 3-methylxanthine. Thus the hypothesis that theophylline is synthesized from xanthine via 3-methylxanthine (Ogutuga & Northcote, 1970*b*) seems to be unlikely.

Table 3 also suggests that xanthine, xanthosine, XMP and hypoxanthine cannot serve as the direct or indirect precursors of 7-methylxanthine. Among them, xanthine and hypoxanthine appear to be totally inactive as the methyl acceptors even under different conditions, because they were well degraded



Scheme 1. *Suggested routes of the biosynthesis of caffeine and related methylxanthines in tea plants*
For details see the Discussion section.

by tea-leaf extracts under the same conditions in which methylxanthines were most actively methylated. Although exogenous hypoxanthine can serve as a good precursor of caffeine biosynthesis *in vivo* (Konishi & Oishi, 1973; Suzuki & Takahashi, 1974), this is the result of nucleotide formation via the pathway of purine salvage. This cannot exclude the possibility that caffeine is synthesized from the purine nucleotides synthesized via the pathways of purine bases *de novo* or via the pathways of nucleic acid catabolism. Further, the possiblity that xanthosine and/or XMP can be methylated under different conditions cannot be ruled out, although they were inactive under the conditions of testing (Table 3). Hence the pathways leading to the synthesis of 7-methylxanthine still remain obscure.

Of perhaps most interest is the mechanism of the two methylation reactions, i.e. 7-methylxanthine to theobromine and theobromine to caffeine. Are they catalysed by the same enzyme or two separate ones? What regulatory mechanism operates in these two reactions? However, at present no definite answers can be given for these questions. Our present results show only the following facts. (1) The two methylation reactions exhibit the same pH optimum and are similarly affected by metal ions and inhibitors (Fig. 1 and Table 2). (2) Estimations of the apparent K_m values show the same K_m values both for theobromine and caffeine formation (Figs. 2 and 3), although these K_m values are likely to be altered on further enzyme purification.

The methods introduced in our present experiments depend on the report of Sanderson (1966). Although these methods did not give good results in the case of coffee plants, extracts from tea leaves with Polyclar AT exhibited methyltransferase activity and xanthine oxidase activity. Besides these enzyme activities, we have also found RNA methylase activity (T. Suzuki & E. Takahashi, unpublished work). Hence it may now be possible to detect and assay other enzymes in tea plants and to answer many of the questions about the metabolic pathways.

There is a good correlation (Fig. 5) between caffeine synthesis and the growth or formation of tea shoots. It is probable that these tissues growing rapidly have active purine nucleotide and nucleic acid metabolism so that the methylating systems involved in caffeine biosynthesis in tea plants appear to be closely associated with purine nucleotide and nucleic acid metabolism. The relation between caffeine synthesis and nucleic acid metabolism has been studied (Bhattacharyya & Ghosh, 1968; Konishi *et al.*, 1972*a*; Suzuki, 1973; Suzuki & Takahashi, 1974). Although little is known about the physiological significance of caffeine formation or the role of caffeine in plants, one of the answers will be obtained from more detailed studies on caffeine metabolism, formation and degradation, relating to purine nucleotide and nucleic acid metabolism.

Caffeine has three methyl groups on the nitrogen atoms of the purine ring. The present paper describes the two methylation reactions, i.e. 7-methylxanthine to theobromine and theobromine to caffeine, in caffeine biosynthesis in tea plants. In addition, the methylation of 1-methylxanthine was shown to result in the formation of theophylline and paraxanthine. Scheme 1 summarizes these studies, and suggests the biosynthetic pathways for caffeine and related methylxanthines. Although similar attempts to extract enzyme from coffee leaves were unsuccessful, Scheme 1 (described for tea plants) presumably describes the events in those plants that produce caffeine, e.g. coffee and cacao. In coffee plants at least, caffeine is synthesized from 7-methylxanthine via theobromine; thus Anderson & Gibbs (1962) demonstrated the same pathways as tea plants described in Scheme 1, except that 7-methylxanthine is synthesized from xanthine. Much less is known about the pathways leading to the formation of 7-methylxanthine.

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References

Anderson, L. & Gibbs, M. (1962) *J. Biol. Chem.* **237**, 1941–1944

Bhattacharyya, A. K. & Ghosh, J. J. (1968) *Biochem. J.* **108**, 121–124

Borek, E. & Srinivasan, P. R. (1965) in *Transmethylation and Methionine Biosynthesis* (Shapiro, S. K. & Schlenk, F., eds.), pp. 115–137, University of Chicago Press, Chicago

Franzke, Cl., Grunert, K. S., Hildebrandt, U. & Griehl, H. (1968) *Pharmazie* **23**, 502–503

Inoue, T. (1971) *Proc. Hoshi Pharm.* **13**, 60–62

Inoue, T. & Adachi, F. (1962) *Chem. Pharm. Bull.* **10**, 1212–1214

Jones, J. W. & Robins, K. (1963) *J. Amer. Chem. Soc.* **85**, 193–201

Keller, H., Wanner, H. & Baumann, T. W. (1972) *Planta* **108**, 339–350

Konishi, S. & Oishi, F. (1973) *Nippon Chagyo-Gijutsu Kyokai Kenkyu-Happyokai* Abstr. 38P

Konishi, S., Inoue, T. & Takahashi, E. (1972*a*) *Plant Cell Physiol.* **13**, 695–702

Konishi, S., Ozasa, M. & Takahashi, E. (1972*b*) *Plant Cell Physiol.* **13**, 365–375

Loomis, W. D. (1969) *Methods Enzymol.* **13**, 555–563

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Ogutuga, D. B. A. & Northcote, D. H. (1970*a*) *J. Exp. Bot.* **21**, 258–273

Ogutuga, D. B. A. & Northcote, D. H. (1970*b*) *Biochem. J.* **117**, 715–720

Preusser, E. (1967) *Biol. Zentralbl.* **86**, 485–494

Preusser, E. (Proiser, E.) & Serenkov, G. P. (1963) *Biokhimiya* **28**, 857–861

Sanderson, G. W. (1966) *Biochem. J.* **98**, 248–252

Suzuki, T. (1972) *FEBS Lett.* **24**, 18–20

Suzuki, T. (1973) *Biochem. J.* **132**, 753–763

Suzuki, T. & Takahashi, E. (1974) *Biochem. J.***146**, 79–85

Wanner, H. & Blaim, K. (1961) *Planta* **56**, 499–510