

Metabolism of Xanthine and Hypoxanthine in the Tea Plant (*Thea sinensis* L.)

By TAKEO SUZUKI and EIICHI TAKAHASHI

Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan

(Received 17 June 1974)

1. The metabolism of xanthine and hypoxanthine in excised shoot tips of tea was studied with micromolar amounts of [2-¹⁴C]xanthine or [8-¹⁴C]hypoxanthine. Almost all of the radioactive compounds supplied were utilized by tea shoot tips by 30h after their uptake. 2. The main products of [2-¹⁴C]xanthine and [8-¹⁴C]hypoxanthine metabolism in tea shoots were urea, allantoin and allantoic acid. There was also incorporation of the label into theobromine, caffeine and RNA purine nucleotides. 3. The results indicate that tea plants can catabolize purine bases by the same pathways as animals. It is also suggested that tea plants have the ability to synthesize purine nucleotides from glycine by the pathways of purine biosynthesis *de novo* and from hypoxanthine and xanthine by the pathway of purine salvage. 4. The results of incorporation of more radioactivity from [8-¹⁴C]hypoxanthine than from [2-¹⁴C]xanthine into RNA purine nucleotides and caffeine suggest that hypoxanthine is a more effective precursor of caffeine biosynthesis than xanthine. The formation of caffeine from hypoxanthine is a result of nucleotide synthesis via the pathway of purine salvage.

Although a number of investigations have been made on the catabolic pathways of purine bases, there appear to be no reports on the metabolism of xanthine and hypoxanthine both in tea and in coffee plants, in spite of the importance of these plants since they contain significant amounts of caffeine and related xanthine bases (Weevers, 1930).

Kalberer (1964, 1965) has revealed that coffee plants can catabolize caffeine to xanthine, which is further metabolized to allantoin, then to allantoic acid and finally to urea and CO₂. Further, Konishi & Oishi (1973) found that urea was a product of xanthine metabolism in tea plants. These studies suggest that both coffee and tea plants have the ability to metabolize xanthine by the same pathways of purine catabolism observed in animals (Hartman, 1970).

Hypoxanthine and xanthine are also known to be metabolized via the purine-salvage pathway. The conversion of hypoxanthine into IMP and of xanthine into XMP is catalysed by the same enzyme hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8) (Murray, 1971).

Although early investigations on caffeine biosynthesis with coffee plants (Anderson & Gibbs, 1962) and with tea plants (Preusser & Serenkov, 1963) have shown that the purine ring of caffeine is synthesized by the same pathways as the purine bases, it is still not known whether the purine ring of caffeine is derived directly from xanthine, or whether caffeine arises from the breakdown of some

preformed nucleotides. Ogutuga & Northcote (1970) have demonstrated that the purine ring of caffeine is derived from the breakdown of nucleic acids. In experiments with plucked tea shoot tips, Konishi & Oishi (1973) have found that hypoxanthine can serve as a precursor for caffeine biosynthesis more effectively than xanthine. This also suggests that caffeine arises from the breakdown of some preformed nucleotides rather than directly from xanthine.

The present paper describes a systematic study on the metabolism of xanthine and hypoxanthine in tea plants. Results are discussed in relation to the possible pathways for xanthine and hypoxanthine metabolism and for caffeine biosynthesis in tea plants.

Materials and Methods

Materials

7-Methylxanthine was prepared as described by Jones & Robins (1963). 3-Methylxanthine was kindly provided by Dr. M. Sekiya, Shizuoka College of Pharmacy, Shizuoka, Japan. 1-Methylxanthine was generously provided by Dr. S. Schwimmer, Western Regional Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, Calif., U.S.A.

[2-¹⁴C]Xanthine (48 mCi/mmol), [8-¹⁴C]hypoxanthine (47 mCi/mmol) and L-[Me-¹⁴C]methionine (53 mCi/mmol) were purchased from Le Commissariat à l'Énergie Atomique, Paris, France. [1-¹⁴C]Glycine

(19.4mCi/mmol) was purchased from Daiichi Pure Chemical Co., Tokyo, Japan. In all feeding experiments the radioactive compounds were diluted with water to give 10 μ Ci in 1 ml of solution.

Plants and feeding experiments

The plant material and the methods of feeding experiments were as described by Suzuki (1973).

Extraction and preparation of acid-soluble materials and RNA nucleotides

The procedure of extraction and preparation of acid-soluble materials and RNA nucleotides was as described by Suzuki (1973).

Radioautography of 14 C-labelled products of acid-soluble materials

The acid-soluble materials, after neutralization with solid KHCO_3 and removal of KClO_4 , were subjected to two-dimensional radioautography to characterize the ^{14}C -labelled products and to assay their radioactivities. Ascending paper chromatography was carried out overnight on Whatman no. 1 paper, with the following solvent systems: (1) butan-1-ol-acetic acid-water (4:1:1, by vol.) followed by ethanol-acetic acid-water (81:5:14, by vol.) (Kalberer, 1965); (2) ethanol-acetic acid-water (81:5:14, by vol.) followed by pyridine-conc. NH_3 (sp.gr. 0.90) (47:3, v/v) (Dickstein *et al.*, 1956); (3) 2-methylpropan-2-ol-butan-2-one-water-dimethylamine (10:10:5:1, by vol.) followed by propan-1-ol-0.1% NH_3 (2:1,v/v). Authentic materials were co-chromatographed on each sheet. Radioactive areas on the paper chromatograms were located by using Fuji X-ray film. Caffeine and authentic purine bases were detected by u.v. quenching (at 253.7nm) and the authentic allantoin, allantoic acid and urea were detected by their reaction with Ehrlich reagents.

By using paper chromatography either in solvent (1) or (2), 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} and radioactivity of each fraction were as described by Suzuki (1973). The identity of the nucleotides was established by their elution positions from the Dowex 1 (formate form) resins and co-chromatography on Whatman no. 1 paper with authentic nucleotides in solvents (4) propan-2-ol-conc. HCl (sp.gr. 1.18)-water (14:3:3,

by vol.) and (5) isobutyric acid-0.5M- NH_3 (5:3, v/v). The radioactive purine nucleotides, after hydrolysis of the pooled fractions from the column with 1M-HCl at 100°C for 1h, were further identified by paper chromatography in solvent (6) methanol-ethanol-water-conc. HCl (50:25:19:6, by vol.).

For a quantitative assay of the ^{14}C -labelled AMP and GMP, u.v.-absorbing fractions corresponding to AMP and GMP were collected from the column. They were then concentrated to a small volume in a rotary evaporator under vacuum, and rechromatographed on Whatman 3MM paper in solvent (4). The positions of the u.v.-absorbing spots on the papers corresponding to AMP and GMP were marked in pencil, cut from the papers, and their radioactivities measured in a Beckman LS-100 liquid-scintillation counter as described by Suzuki (1973).

Extraction and measurement of caffeine

Caffeine was extracted, isolated and measured by the methods of Konishi *et al.* (1972). Radioactivity of caffeine was determined in a Beckman LS-100 liquid-scintillation counter as described by Konishi *et al.* (1972).

Results

Preliminary feeding experiments with ^{14}C -labelled precursors

The synthesis of caffeine and purine nucleotides of RNA in tea shoot tips was examined by using various ^{14}C -labelled precursors (Table 1). In each case, 5 μ Ci of the radioactive precursor in 0.5 ml of solution was supplied to 2.0-2.1 g of four shoot tips (detached from 80-day-old seedlings) which were then allowed to incubate in water for 9h. The results of these feeding experiments are summarized in Table 1.

Among the possible precursors of caffeine biosynthesis, L-[Me- ^{14}C]methionine was the most effective. Significant amounts of radioactivity from [8- ^{14}C]hypoxanthine were also incorporated into caffeine, whereas only very small amounts of radioactivity from [2- ^{14}C]xanthine were incorporated, indicating that the purine ring of caffeine is not derived directly from xanthine. This agrees with the report of Konishi & Oishi (1973) on caffeine biosynthesis.

[1- ^{14}C]Glycine was used to confirm the purine biosynthesis *de novo*; more radioactivity was incorporated from [1- ^{14}C]glycine into AMP and GMP of RNA than from [2- ^{14}C]xanthine or from [8- ^{14}C]hypoxanthine, indicating that a significant synthesis of purine bases *de novo* takes place in tea shoot tips. In contrast with this, when tea shoot tips were incubated with [8- ^{14}C]hypoxanthine, a considerable amount of radioactivity was incorporated into the

Table 1. Incorporation of ¹⁴C-labelled precursors into caffeine and adenylic acid and guanylic acid of RNA in tea shoot tips

Each of four excised shoot tips (2.0–2.1 g fresh wt.) of 80-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 h, followed by incubation for a 9 h period in water in a 50 ml Erlenmeyer flask, and was then processed as described in the Materials and Methods section.

Precursor	Specific radio-activity (mCi/mmol)	¹⁴ C incorporated (c.p.m./shoot) into		
		Caffeine	AMP	GMP
L-[Me- ¹⁴ C]Methionine	53	55 000	—	—
[1- ¹⁴ C]Glycine	19	2500	45 300	20 500
[2- ¹⁴ C]Xanthine	48	320	230	120
[8- ¹⁴ C]Hypoxanthine	47	14 600	600	15 300

GMP of RNA, whereas only small amounts of radioactivity from [2-¹⁴C]xanthine were incorporated.

The reason for the large amount of radioactivity incorporated into GMP compared with that into AMP, when tea shoot tips were labelled with [8-¹⁴C]-hypoxanthine, is not known, but it was confirmed that the radioactivity of GMP, after hydrolysis with 1 M-HCl at 100°C for 1 h and rechromatography on Whatman no. 1 paper in solvent system (6), was present in guanine only.

Analysis of the metabolites produced from xanthine and hypoxanthine

The results of preliminary feeding experiments indicated that hypoxanthine can serve as a precursor both of caffeine formation and of purine nucleotide synthesis more effectively than xanthine. Further, Konishi & Oishi (1973) found that urea was a product of xanthine metabolism in tea plants. These facts suggest that in tea plants xanthine is catabolized in a manner similar to that known for other organisms (Hartman, 1970). Thus to confirm this [2-¹⁴C]-xanthine and [8-¹⁴C]hypoxanthine were supplied to tea shoot tips respectively, and the ¹⁴C-labelled products of acid-soluble extracts were examined after a 10h experimental period. The results of these experiments are summarized in Fig. 1. In both cases, urea, xanthine, allantoin and allantoic acid are the major products of xanthine and hypoxanthine metabolism. These results are similar to those reported by Kalberer (1964, 1965). However, in addition to these major products, theobromine was obtained as an important product of xanthine and hypoxanthine metabolism. In contrast caffeine was only obtained from hypoxanthine. Further, in addition to these identified products, there were also minor products (Fig. 1), which have not yet been identified.

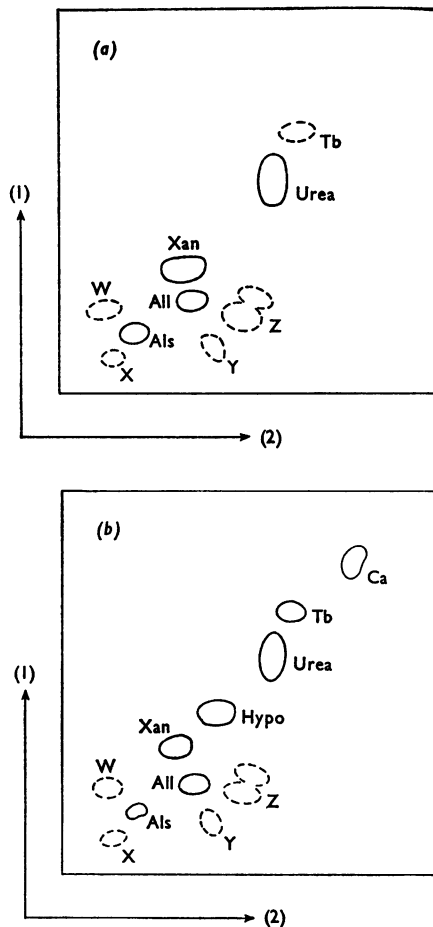


Fig. 1. Tracing of two-dimensional radioautograms of radioactive products from tea shoot tips 8h after the absorption of (a) [2-¹⁴C]xanthine (10 μCi in 1 ml of solution) and of (b) [8-¹⁴C]hypoxanthine (10 μCi in 1 ml of solution)

The neutralized supernatant-fluid extracts were subjected to two-dimensional ascending chromatography on Whatman no. 1 paper, with butan-1-ol-acetic acid-water (4:1:1, by vol.) in direction (1) and ethanol-acetic acid-water (81:5:14, by vol.) in direction (2). Abbreviations: Xan, xanthine; Hypo, hypoxanthine; All, allantoin; Als, allantoic acid; Tb, theobromine; Ca, caffeine; W, X, Y, Z, unknown substances.

Sequence of incorporation of radioactivity into products of xanthine and hypoxanthine metabolism in tea shoot tips

The time-course for incorporation of radioactivity from [2-¹⁴C]xanthine and from [8-¹⁴C]hypoxanthine into the metabolites was investigated by feeding tea shoot tips with [2-¹⁴C]xanthine (10 μCi in 1 ml of

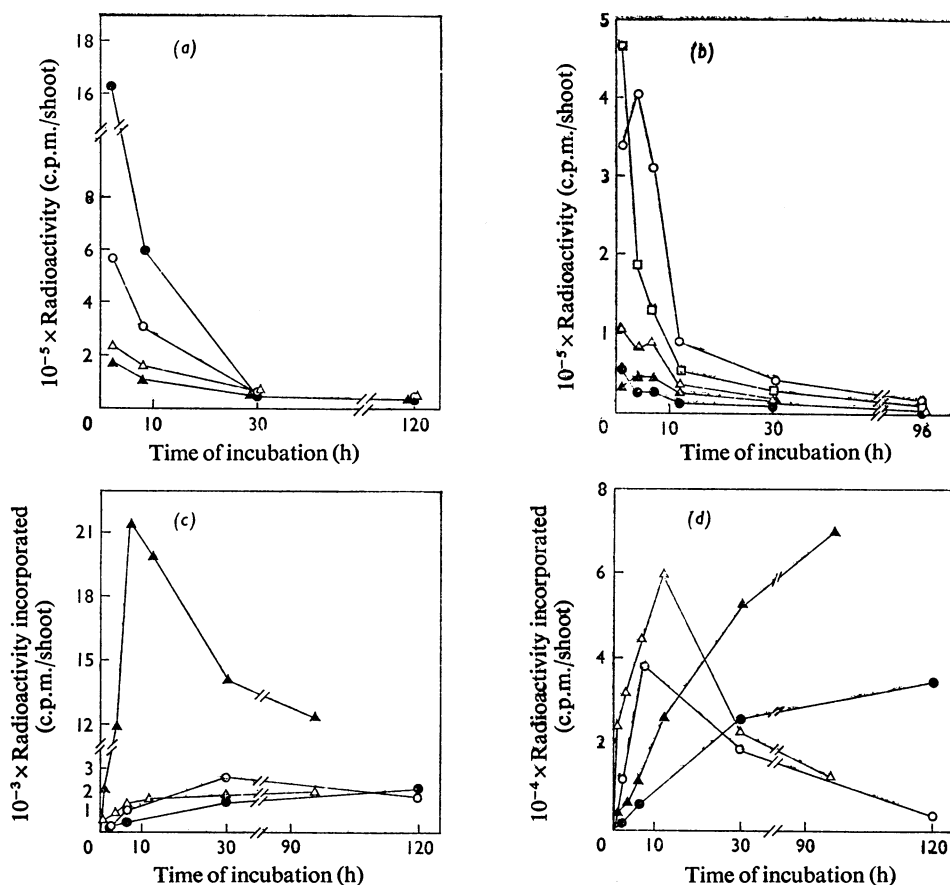


Fig. 2. Metabolism of xanthine and hypoxanthine in tea shoot tips

(a) Distribution of radioactivity among xanthine (●), allantoin (Δ), allantoic acid (▲) and urea (○), when tea shoot tips were fed with $10 \mu\text{Ci}$ of $[2\text{-}^{14}\text{C}]$ xanthine (48 mCi/mmol). (b) Distribution of radioactivity among hypoxanthine (□), xanthine (●), allantoin (Δ), allantoic acid (▲) and urea (○), when tea shoot tips were fed with $5 \mu\text{Ci}$ of $[8\text{-}^{14}\text{C}]$ hypoxanthine (47 mCi/mmol). (c) Incorporation of radioactivity from $[2\text{-}^{14}\text{C}]$ xanthine (○, ●) and from $[8\text{-}^{14}\text{C}]$ hypoxanthine (Δ, ▲) into the AMP (○, Δ) and GMP (●, ▲) of RNA in tea shoot tips. (d) Incorporation of radioactivity from $[2\text{-}^{14}\text{C}]$ xanthine (○, ●) and from $[8\text{-}^{14}\text{C}]$ hypoxanthine (Δ, ▲) into theobromine (○, Δ) and caffeine (●, ▲) in tea shoot tips. Batches of four excised shoot tips ($2.0\text{--}2.1 \text{ g}$ fresh wt.) were each fed with $10 \mu\text{Ci}$ of $[2\text{-}^{14}\text{C}]$ xanthine within 2 h or within $5 \mu\text{Ci}$ of $[8\text{-}^{14}\text{C}]$ hypoxanthine within 1 h and then incubated in water in 50 ml Erlenmeyer flasks for various periods. Other methods are described in the text.

solution) for a 2 h incubation period, or with $[8\text{-}^{14}\text{C}]$ hypoxanthine ($5 \mu\text{Ci}$ in 0.5 ml of solution) for a 1 h incubation period before analysis. Almost all of the radioactive compounds supplied were taken up during this incubation period. Incubation of the tips in water was then continued for various periods of time before subsequent analysis. The results of these feeding experiments are shown in Fig. 2.

Catabolism of xanthine and hypoxanthine. Fig. 2(a) and 2(b) show the time-course for the distribution of radioactivity among hypoxanthine, xanthine, urea, allantoin and allantoic acid in tea shoot tips after the uptake of $[2\text{-}^{14}\text{C}]$ xanthine or of $[8\text{-}^{14}\text{C}]$ hypoxanthine.

In both cases the radioactivities of the radioactive compounds supplied were rapidly converted into urea, allantoin and allantoic acid; the radioactivities of these compounds also decreased rapidly, and only small amounts of radioactivity remained after a 30 h period, suggesting that active degradation of xanthine and hypoxanthine takes place in tea plants by the same pathways as purine catabolism in animals.

Purine nucleotide synthesis. Fig. 2(c) shows the time-course for incorporation of radioactive xanthine and hypoxanthine into purine nucleotides of RNA after the uptake of $[2\text{-}^{14}\text{C}]$ xanthine ($10 \mu\text{Ci}$) or of $[8\text{-}^{14}\text{C}]$ hypoxanthine ($5 \mu\text{Ci}$). When tea shoot tips

were fed with [8-¹⁴C]hypoxanthine, radioactivity was rapidly incorporated into AMP and GMP of RNA. Again, as described above, large amounts of the RNA purine nucleotide radioactivity derived from [8-¹⁴C]hypoxanthine was found in GMP, compared with small amounts of radioactivity in AMP. The radioactivity in GMP derived from [8-¹⁴C]hypoxanthine reached a maximum value at 7h, and then decreased rapidly, whereas that in AMP continued to rise slowly during the experimental periods.

In contrast with the results with [8-¹⁴C]hypoxanthine the radioactivity in GMP derived from [2-¹⁴C]xanthine, after a short lag period, increased slightly throughout the incubation periods, whereas the radioactivity in AMP derived from [2-¹⁴C]xanthine reached a maximum value at 30h and then decreased slowly. The incorporation of radioactivity from [8-¹⁴C]hypoxanthine into RNA purine nucleotides was much greater than that from [2-¹⁴C]xanthine, although twice as much [2-¹⁴C]xanthine (48 mCi/mmol) was absorbed as [8-¹⁴C]hypoxanthine (47 mCi/mmol).

Theobromine and caffeine synthesis. The time-course for incorporation of radioactive xanthine and hypoxanthine into theobromine and caffeine after the uptake of [2-¹⁴C]xanthine (10 μCi) or of [8-¹⁴C]hypoxanthine (5 μCi) is shown in Fig. 2(d). The incorporation of radioactivity from [8-¹⁴C]hypoxanthine into theobromine and caffeine was faster and greater than that from [2-¹⁴C]xanthine. In both feeding experiments, there was a greater incorporation of radioactivity into theobromine than into caffeine during the early periods of incubation, and the subsequent increase of radioactivity in caffeine was accompanied by a decrease of radioactivity in theobromine. Similar results were obtained by feeding tea shoot tips with [¹⁴C]methylamine (Suzuki, 1973).

Discussion

The results of the present experiments demonstrate the following two important aspects of purine catabolism and caffeine biosynthesis in tea plants. (1) Tea plants can catabolize xanthine and hypoxanthine by the same pathways as purine catabolism in animals. (2) Caffeine arises from some purine nucleotides synthesized by the pathways of purine synthesis *de novo* or derived from the catabolic pathways of nucleic acids. Exogenous hypoxanthine is utilized in preference to exogenous xanthine for the synthesis of RNA purine nucleotides and caffeine. These results are comparable with those observed in tea callus tissue fed with L-[Me-¹⁴C]methionine, in that caffeine arises as a result of nucleic acid breakdown (Ogutuga & Northcote, 1970).

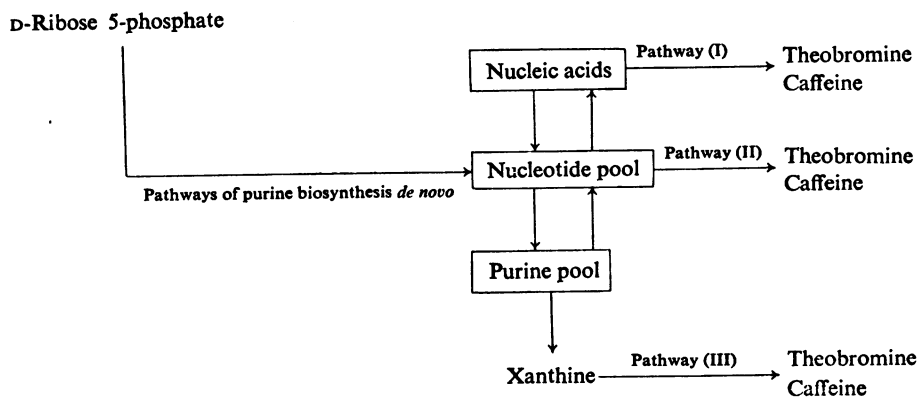
The usual fate of purines derived from nucleic acid catabolism is further breakdown of hypoxanthine and

xanthine into uric acid, allantoin, allantoic acid, urea and CO₂ (Hartman, 1971). The form in which it is finally excreted depends on the presence or absence of the enzymes involved. The results of the present experiments clearly show that urea, allantoin and allantoic acid are all important products of xanthine and hypoxanthine metabolism in tea plants (Fig. 1). Further, the rapid decreases of radioactivity in the radioactive compound supplied, and of the metabolic intermediate allantoin, allantoic acid and urea (Figs. 2a and 2b), suggest that CO₂ is the end product of purine catabolism in tea plants although radioactivity incorporation into CO₂ was not confirmed in the present studies. However, only CO₂ evolution can account for the loss of the net radioactivity incorporated. Therefore it is concluded that active degradation of hypoxanthine and xanthine by these pathways takes place in tea plants.

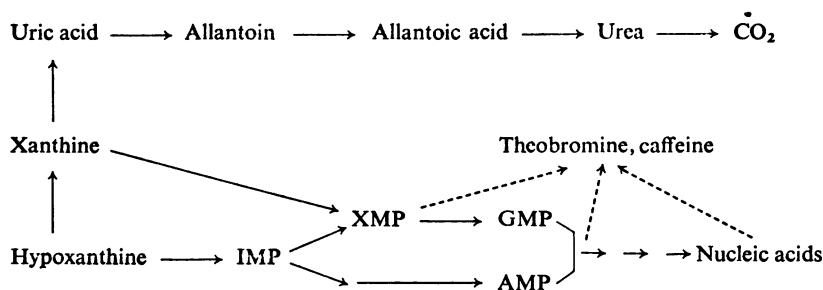
The results also argue for the synthesis of purine nucleotides via the pathway of purine salvage in tea shoot tips, even though tea shoot tips can synthesize purine nucleotides *de novo* (Table 1). The utilization of hypoxanthine for the synthesis of nucleotides is much greater than that of xanthine (Fig. 2c). This difference is exaggerated in these studies because twice as much [2-¹⁴C]xanthine was taken up as [8-¹⁴C]hypoxanthine. The conversion of hypoxanthine into IMP and of xanthine into XMP are catalysed by the same enzyme, hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8).

The incorporation of more radioactivity from [8-¹⁴C]hypoxanthine into GMP than into AMP, for which no reason can be given, suggests that nucleic acid synthesis utilizing IMP derived from hypoxanthine and utilizing IMP synthesized by purine synthesis *de novo* may proceed via two different pathways in the organized systems of tea shoots, because the synthesis of purine nucleotides *de novo* resulted in the incorporation of more radioactivity into AMP than into GMP (Table 1). However, in contrast with the results with [8-¹⁴C]hypoxanthine-feeding experiments, more radioactivity was incorporated from [2-¹⁴C]xanthine into AMP than into GMP during the first 30h incubation period (Fig. 2c). As the first step of xanthine utilization for the synthesis of purine nucleotides is the formation of XMP from xanthine, there appear to be several steps of interconversion of purine nucleotides whereby nucleic acids are synthesized, but these steps have not yet been further studied.

Hypoxanthine is also shown to be utilized in preference to xanthine for the synthesis of theobromine and caffeine (Table 1; Fig. 2d). Theobromine has been shown to be the immediate precursor of caffeine (Ogutuga & Northcote, 1970; Suzuki, 1973). Scheme 1 is a representation of possible pathways of theobromine and caffeine biosynthesis. The results of [2-¹⁴C]xanthine-feeding experiments (Figs. 1 and 2d)



Scheme 1. Possible pathways of theobromine and caffeine biosynthesis



Scheme 2. Metabolism of xanthine and hypoxanthine in tea shoot tips

indicated that Pathway (III) is unlikely to be the major route as demonstrated by Ogutuga & Northcote (1970).

The results of [8-¹⁴C]hypoxanthine-feeding experiments (Figs. 2c and 2d) argue for Pathways (I) and (II). Purine nucleotides can be synthesized from hypoxanthine via the pathway of purine salvage. The incorporation of more radioactivity from [8-¹⁴C]-hypoxanthine into GMP than into AMP indicates that the conversion of IMP into XMP and into GMP is greater than that into adenylosuccinic acid and into AMP. Although Ogutuga & Northcote (1970) demonstrated Pathway (I), one cannot decide which pathway is more likely on the basis of the results of our experiments, since it seems that radioactivity is incorporated into theobromine, caffeine and the RNA purine nucleotides at almost the same rate (Figs. 2c and 2d). Also the possibility that caffeine is synthesized from XMP is not excluded, although only small amounts of radioactivity are incorporated into theobromine and caffeine (Fig. 2d), and the conversion of xanthine into XMP appears to be small, judging from the incorporation of [2-¹⁴C]-

xanthine into the RNA purine nucleotides (Fig. 2c). It is likely therefore that theobromine and caffeine can arise from XMP or the other purine nucleotides, either synthesized 'de novo' by the pathways or derived from the catabolic pathways of nucleic acids. More detailed studies of the methylating systems in tea plants that produce theobromine and caffeine will be described in the following paper (Suzuki & Takahashi, 1974).

From the present studies it appears that the metabolism of xanthine and hypoxanthine in tea plants occurs as summarized in Scheme 2. Metabolism of [8-¹⁴C]hypoxanthine and [2-¹⁴C]xanthine by these pathways would lead to the observed distribution of the radioactivity among the isolated products. Theobromine and caffeine might arise as a result of purine nucleotide synthesis via the pathway of purine salvage. In addition, Kalberer (1964, 1965) has shown that coffee plants can catabolize caffeine to xanthine, which is further metabolized to allantoin, allantoic acid, urea and CO₂, and Scheme 2 (described for tea plants) presumably also describes the events in coffee plants.

This and other papers (Suzuki, 1972, 1973; Suzuki & Takahashi, 1974) are parts of the doctoral thesis submitted by T.S. in partial fulfilment of the requirements of the Ph.D. degree in the Department of Agricultural Chemistry, Kyoto University. We are grateful to Dr. M. Sekiya for a gift of 3-methylxanthine and Dr. S. Schwimmer for a gift of 1-methylxanthine. We also thank Dr. S. Konishi of the Department of Agricultural Chemistry, Shizuoka University, Shizuoka, Japan for useful comments, Dr. K. Iwai of the Research Institute for Food Science, Kyoto University, Uji, Kyoto, Japan for helpful discussions, and Dr. S. M. J. Ksycki of Notre Dame College, St. Louis, Mo., U.S.A. for helpful comments and for reviewing the manuscript.

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