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Metabolism of Methylamine in the Tea Plant (*Thea sinensis* L.)

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1. The metabolism of methylamine in excised shoot tips of tea was studied with micromolar amounts of [^{14}C]methylamine. Of the [^{14}C]methylamine supplied 57% was utilized by tea shoots during the 10h experimental period. 2. The main products of [^{14}C]methylamine metabolism in tea shoots were serine, γ-glutamylmethylamide, theobromine, caffeine and CO_2. There was also incorporation of the label into glutamate, aspartate, RNA purine nucleotides and *S*-adenosylmethionine. 3. The formation of methylamine from γ-glutamylmethylamide was confirmed by feeding tea shoots with γ-glutamyl[^{14}C]-methylamide. The products of γ-glutamyl[^{14}C]methylamide metabolism in tea plants were serine, theobromine, caffeine, glutamate and aspartate. 4. The results indicate that the oxidation of methylamine to formaldehyde is the first step of methylamine utilization. Labelled formaldehyde released by the metabolism of methylamine leads to the incorporation of the label into metabolites on the C_1 pathways of this compound. It is also suggested that formaldehyde is further oxidized via formate to CO_2. 5. The role of γ-glutamylmethylamide in methylamine metabolism in tea plants is discussed. 6. Results support the view that theobromine is the immediate precursor of caffeine.

Both in tea and in coffee plants, methylamine can serve as a precursor of caffeine (Preusser & Serenkov, 1963; Preusser, 1967). However, very little is known about the metabolism of methylamine in higher plants. Scheffer *et al.* (1968) demonstrated that methylamine can act as a C_1 precursor in sunflower plants (*Helianthus annuus* L.).

Although the literature on alkyl amine metabolism is not extensive, there is now considerable evidence that the oxidation of methylamine to formaldehyde is the first step of methylamine utilization. Amine oxidases from *Pseudomonas* AM_1 (Eady & Large, 1968), *Aspergillus nidulans* (Adachi & Yamada, 1969) and *Pseudomonas* MS (Kung & Wagner, 1970) have been studied. These studies indicate that an amine oxidase produces formaldehyde and ammonia from methylamine by the following reaction:

$$CH_3NH_2 + H_2O + O_2 \rightarrow HCHO + NH_3 + H_2O_2$$

The formaldehyde formed is further oxidized to CO_2, providing a source of energy in micro-organisms growing on methylamine as the sole carbon source (Eady & Large, 1968; Kung & Wagner, 1970).

Formaldehyde is also known to be easily metabolized as a C_1 unit. Serine (Siegel & Lafaye, 1950) and methionine (Nakao & Greenberg, 1955, 1958) are products of such formaldehyde metabolism. In experiments with plucked tea shoot tips, Konishi *et al.* (1969) reported the conversion of methylamine into serine. This suggests that methylamine is oxidized to formaldehyde, and is incorporated into serine through the C_1 pathway in tea plants.

An unusual amino acid, γ-glutamylmethylamide, was shown to be a product of methylamine metabolism in tea plants (Konishi & Takahashi, 1966). Kung & Wagner (1969) also identified this compound as a product of methylamine metabolism by *Pseudomonas* MS and demonstrated its intermediary importance in the metabolism of methylamine. Further, in recent studies of γ-glutamylmethylamide metabolism in tea plants, Konishi *et al.* (1972*a*,*b*) have reported that the *N*-methyl carbon atom of γ-glutamylmethylamide is converted into the purine ring carbon atoms and the *N*-1-, *N*-3- and *N*-7-methyl carbon atoms of caffeine and the RNA purine nucleotides.

The present paper describes experiments in which [^{14}C]methylamine was fed to excised tea shoot tips, and the subsequent fate of this compound was then investigated. γ-Glutamyl[^{14}C]methylamide and DL-[3-^{14}C]serine were also used to obtain information on the metabolism of methylamine. Results are discussed in relation to the possible pathways for methylamine metabolism in tea plants.

Materials

Chemicals

S-Adenosyl-L-methionine was purchased from Boehringer, Mannheim, Germany. Polyclar AT [polyvinylpyrrolidine (insoluble)] was obtained from

Gokyo Sangyo Co., Osaka, Japan. 7-Methylxanthine was kindly provided by Dr. K. Nakajima, Department of Food Technology, Kyoto University, Kyoto, Japan. 3-Methylxanthine was generously provided by Dr. K. Ina, Department of Agricultural Chemistry, Shizuoka University, Iwata, Shizuoka, Japan.

[^{14}C]Methylamine hydrochloride (17mCi/mmol) was purchased from Le Commissariat à l'Énergie Atomique, Paris, France. DL-[3-^{14}C]Serine (38.9mCi/mmol) was obtained from Daiichi Pure Chemicals Co., Osaka, Japan. γ-Glutamyl[^{14}C]methylamide was obtained by biosynthesis from [^{14}C]methylamine with tea seedlings (20-day-old) and isolated as described by Konishi & Takahashi (1966). In all feeding experiments the radioactive compounds were diluted with water to give 10μCi in 1ml of solution.

Plants

Seeds of tea (*Thea sinensis* L.) were surface-sterilized by dipping them in 0.004% methoxyethylmercuric chloride solution for 4h, and soaked overnight in running water. After this soaking period the seeds were sown in pots of moist vermiculite and allowed to germinate and grow in normal daylight in a greenhouse maintained at 25°C during the day and at 15°C at night. The shoot tips (about 6cm long), comprising the bud, three leaves and the included stem, were excised from 80-day-old seedlings except as otherwise noted.

Methods

Feeding experiments

Four shoots (2.5–2.6g fresh wt.) were used except where otherwise stated. Cut ends of excised shoots were washed well with water and placed in small vials containing the required quantity of radioactive compound. Experiments were carried out at 25°C in normal daylight in a greenhouse. Almost all the radioactive compound in 0.5ml of solution was taken up within 1h. After absorption of the radioactive compound, shoots were transferred to water in 50-ml Erlenmeyer flasks and incubated for various periods. At the end of the incubation period, shoots were placed in a deep-freezer and kept frozen until required for extraction.

Preparation and fractionation of acid-soluble materials, lipid and RNA nucleotides

The frozen shoots were cut into small pieces, and ground with 10ml of cold 0.2M-$HClO_4$ and about 1g of washed sea sand in a chilled mortar. After centrifugation (7000g; 15min) the residue was re-extracted with 5ml of cold 0.2M-$HClO_4$. The washings were combined with the supernatant. The acid-insoluble residue was used for the separation of RNA fractions as described below. The combined supernatants were adjusted to pH6.5 with solid $KHCO_3$. A further centrifugation yielded the extract referred to as 'acid-solubles' in Table 1.

Acid-soluble materials were separated into four fractions, namely basic amino acids and amines, cationic (mainly neutral and acidic amino acids), anionic (mainly organic acids) and neutral (mainly sugars), by use of ion-exchange resins. Acid-soluble materials were first passed through a column (6cm × 1cm) of the sulphonic resin Dowex 50W (X8; Na^+ form) to remove the unchanged [^{14}C]methylamine. The column was washed well with water, then the effluent was passed through a column of the sulphonic resin Amberlite IR-120 (H^+ form). The resulting effluent was passed through a column of the weakly basic resin Amberlite IR-45 (OH^- form). The effluent from this column contained the sugars referred to in Table 1. The Amberlite IR-120 column was eluted with 2M-NH_3. This eluate contained the neutral and acidic amino acids referred to in Table 1, but also contained free bases, including caffeine and related xanthine bases. Organic acids were eluted from the Amberlite IR-45 resin with 2M-$(NH_4)_2CO_3$.

The insoluble residue was successively washed with 2×40ml of 95% (v/v) ethanol, 40ml of ethanol–chloroform (3:1, v/v), 3×30ml of diethyl ether, and finally dried *in vacuo*. The combined supernatant fluids are referred to as 'lipid material' in Table 1. The dry powder was subjected to alkaline hydrolysis of RNA with 0.5M-KOH at 37°C for 20h as described by Osawa *et al.* (1958). Components of the alkaline hydrolysates of RNA were separated by ion-exchange chromatography as described below.

A sample from each fraction was used to measure radioactivity. Radioactivity was determined in a Kobe Kogyo Corp. type PC-26 gas-flow counter after samples (1ml) of the fractions had been spotted on nickel-plated steel planchets and dried under an i.r. lamp. Measurements were corrected for background counts.

Elution and paper chromatography of methylamine

Basic amino acids and amines were eluted from the Dowex 50W (Na^+ form) column with 0.1M-NaCl. The effluent was collected in 10ml fractions. A sample (0.1ml) from each fraction was used for radioactivity assay, as described above. The identity of methylamine was established by co-chromatography on Whatman no. 1 paper with authentic methylamine in (1) butan-1-ol–acetic acid–water (2:1:1, by vol.), (2) phenol–water (4:1, v/v) and (3) butan-1-ol–conc. HCl (sp.gr. 1.18)–water (7:2:1, by vol.) as the solvent systems. Radioactive areas were detected by scanning the paper chromatograms with a Packard 4Pi Actigraph (model 7201) instrument, and the position of the authentic methylamine spot was revealed by the ninhydrin reaction.

Radioautography of ^{14}C-labelled amino acids and methylated xanthines

The 2M-NH_3 eluates from the Amberlite IR-120 (H^+ form) resin were subjected to two-dimensional radioautography to characterize the ^{14}C-labelled amino acids and methylated xanthines and to assay their radioactivities. Paper chromatography was carried out overnight by ascending development on Whatman no. 1 paper, with the following solvent systems: (4) phenol–water (4:1, v/v) followed by butan-1-ol–acetic acid–water (4:1:1, by vol.); (5) phenol–formic acid–water (500:13:167, by vol.) followed by 2-methylpropan-2-ol–butan-2-one–conc. NH_3 (sp.gr. 0.90)–water (4:3:1:2, by vol.) (Kung & Wagner, 1969); (6) butan-1-ol–acetone–water–dimethylamine (20:20:10:3, by vol.) followed by 2-methylpropan-2-ol–butan-2-one–formic acid–water (8:6:6:3, by vol.). Caffeine and theobromine were further identified by two-dimensional paper chromatography in solvent system (7), ethanol–acetic acid–water (81:5:14, by vol.) followed by pyridine–conc. NH_3 (47:3, v/v) (Dickstein *et al.*, 1956). Appropriate amino acids and methylated xanthines were co-chromatographed on each sheet as authentic materials. Radioactive areas on the paper chromatograms were located by radioautography by using Fuji X-ray film. Caffeine and authentic methylated xanthines were detected by u.v. quenching (253.7nm) and amino acids were detected by their reaction with ninhydrin sprays.

For measurement of radioactivity of the ^{14}C-labelled products, two-dimensional paper chromatograms in solvent (4) were employed. Radioactive areas on the paper chromatograms were located by radioautography, 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/l of toluene. Measurements were made in a Nuclear-Chicago type 6804 liquid-scintillation counter. Measurements were corrected for background counts.

Column and paper chromatography of alkaline hydrolysates of RNA

The alkaline hydrolysates of RNA, after removal of DNA, proteins, cellulose, sea sand and $KClO_4$, were separated by chromatography on a column (20cm×0.6cm) of the strongly basic resin Dowex 1 (X2; formate form; 200–400 mesh) with elution by a gradient of 1–4M-formic acid, by the method of Hurlbert *et al.* (1954) as modified by Osawa *et al.* (1958). The effluent was collected in 3ml fractions. The E_{260} of each fraction was measured with a Shimazu QV-50 spectrophotometer. Radioactivity was assayed in a gas-flow counter after portions (0.5ml) of the fractions had been dried on nickel-plated steel planchets as described above. The identity of the nucleotides was established by their elution positions from the Dowex 1 (formate form) resins and by co-chromatography on Whatman no. 1 paper with authentic nucleotides in solvents (8) propan-2-ol–conc. HCl–water (14:3:3, by vol.), (9) methanol–conc. HCl–water (7:2:1, by vol.) and (10) isobutyric acid–0.5M-NH_3 (5:3, v/v). Detection of the radioactive areas on the paper chromatograms was performed as follows. The position of the u.v.-absorbing spot on the paper was marked in pencil, strips were cut into sections and transferred to vials containing 10ml of the toluene-based scintillator solution, and then the radioactivities were measured, as described above.

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 (8). The positions of the u.v.-absorbing spots on the papers corresponding to AMP and GMP were marked in pencil, then cut from the papers and transferred to vials containing 10ml of the toluene-based scintillator solution. Their radioactivities were assayed as described above.

Extraction of S-adenosylmethionine

A modification of the method of Shapiro & Ehninger (1966) was used. All operations were carried out at 4°C. The frozen shoots were cut into small pieces, and ground with 5–6vol. of 1.5M-$HClO_4$, an equal weight of washed Polyclar AT and about 1g of washed sea sand in a chilled mortar. The homogenate was centrifuged at 9000*g* for 20min. The residue was washed with about 10ml of ice-cold water, and then the pH of the combined supernatants was adjusted to 6.5 with solid $KHCO_3$. This mixture was centrifuged at 9000*g* for 20min. *S*-Adenosylmethionine was isolated from the supernatant fluid by chromatography on a column (5cm× 1cm) of Dowex 50W (X8; Na^+ form).

Displacement and t.l.c. of S-adenosylmethionine

The column-chromatographic method of Shapiro & Ehninger (1966) was used. The column was washed with 50–100ml of water and then eluted with 0.1M-NaCl; 30 10ml fractions were collected. Radioactivities of the fractions were assayed in a gas-flow counter as described above. The column was washed with 50ml of water and then eluted with 6M-HCl; fractions of 9.0ml were collected. Radioactivities of the fractions were determined in a Beckman LS-100 scintillation counter. The counting solution and the methods used for radioactive assay were as described by Dodd & Cossins (1969).

S-Adenosylmethionine isolated by the Dowex 50W (X8; Na^+ form) column was identified by chromatography on silica-gel plates (Eastman Chromagram Sheets 6060) by using the following solvent systems: (11) butan-1-ol–acetic acid–water (12:3:5, by vol.); (12) butan-1-ol–ethanol–propionic acid–water (10:5:2:5, by vol.); (13) ethanol–acetic acid–water (65:1:34, by vol.); (14) 2,4-dimethylpyridine–2,4,6-trimethylpyridine–water (6:5:5, by vol.) (Zappia *et al.*, 1969). The position of the sulphonium compound was detected by u.v. quenching and by ninhydrin reaction. Radioactive areas on chromatograms were detected by scanning with a Packard 2Pi Actigraph (model 7201), or by radioautography with X-ray films.

Results

Metabolism of [^{14}C]methylamine by excised tea shoot tips

Five shoot tips, consisting of three leaves, harvested from rapidly growing 70-day-old tea seedlings, were used. During the 10h experimental period, the shoots metabolized approx. 57% of the [^{14}C]methylamine supplied (Table 1). This utilization resulted in methylamine carbon being incorporated into all the fractions investigated. The value for 'other compounds' (incorporation into residue or in the CO_2 evolved) was obtained by subtracting the radioactivity in the remainder of the fractions from the radioactivity supplied. This value was 36% of the ^{14}C incorporated. Also, considerable amounts of radioactivity were incorporated into the neutral and acidic amino acid fraction (which was also found to contain theobromine and caffeine in subsequent experiments, as mentioned in the next section) and into lipid material. IR-120 (H^+ form) resin were examined after a 10h experimental period. The radioactive components of the eluate were separated by two-dimensional paper chromatography by each of the systems described in the Methods section, and were located by radioautography. Results (Fig. 1) are similar to those reported by Konishi *et al.* (1969), that is, γ-glutamylmethylamide and serine are the major products of methylamine metabolism. However, in addition to these two products, caffeine and theobromine, and two minor products, glutamate and aspartate, were obtained. By using paper chromatography either in solvents (5) or (6), glycine was well separated from serine, but in neither case was the labelling of glycine confirmed. The identification of caffeine and theobromine was checked by paper chromatography in solvent system (7). On radioautograms from solvent (7) a very weakly radioactive spot coinciding with authentic 7- Smaller amounts of ^{14}C were present in the sugars and in the organic acids.

Metabolic labelling of amino acids and methylated xanthines

The neutral and acidic amino acid fraction was the major product of the acid-soluble fractions (Table 1). Further, important components of tea plants, caffeine and related xanthines (i.e. precursors of caffeine) were also contained in this fraction. In experiments with plucked tea shoot tips, Konishi *et al.* (1969) reported a conversion of methylamine carbon into γ-glutamylmethylamide, serine and two other unknown compounds. In the present studies, [^{14}C]-methylamine (20μCi) was supplied to tea shoot tips, and the ^{14}C-labelled amino acids and methylated xanthines in the 2M-NH_3 eluate from the Amberlite

Table 1. *Utilization of [^{14}C]methylamine by excised tea shoot tips*

Five excised shoot tips (2.4g fresh wt.) of 70-day-old seedlings were placed with their cut ends in a small vial containing 20μCi of [^{14}C]methylamine in 2ml of solution for 4h, followed by incubation for a 6h period in water in a 50ml Erlenmeyer flask, and were then processed as described in the Methods section.

Fraction	10^{-6} × Radioactivity (c.p.m.)	% of ^{14}C incorporated
Acid solubles	3.804	57.6
Neutral and acidic amino acids, bases	0.710	10.8
Basic amino acids	Negligible	—
Sugars	0.144	2.2
Organic acids	0.092	1.4
Methylamine	2.858	43.2
Lipids	0.413	6.3
Other compounds (residue and CO_2)	2.383	36.1
Total ^{14}C incorporated	6.600	—
Non-incorporated [^{14}C]methylamine	Negligible	—
% of [^{14}C]methylamine utilized	—	57

methylxanthine was also observed. In addition, there were also minor radioactive spots (Fig. 1), which have not yet been identified.

Metabolic labelling of purines

Previous work on caffeine biosynthesis (Anderson & Gibbs, 1962; Preusser & Serenkov, 1963) established that the purine ring of caffeine is synthesized by the same pathways as the purine bases (Buchanan & Hartman, 1959). Although Preusser (1967) demonstrated that the incorporation of methylamine carbon into caffeine is due to methylation of the caffeine precursors, it appears likely that the formation of formaldehyde from methylamine results in incorporation of the methylamine carbon into the nucleic purines as well as into the purine ring of caffeine through the C_1 pathway. In experiments with coffee leaf squares, Anderson & Gibbs (1962) reported the incorporation of formaldehyde carbon into C-2 and C-8 of caffeine. In the present studies, [^{14}C]methylamine (20μCi) was fed to tea shoot tips (6g fresh wt.) of 85-day-old seedlings, and the incorporation of ^{14}C into RNA was examined after incubation in water in a 50ml Erlenmeyer flask for 4 days. The total RNA nucleotides prepared from shoot tips were chromatographed on an anion-exchange column (Fig. 2). Five peaks of radioactivity appeared: two apparently coinciding with AMP and GMP, two emerging just before CMP, and one before GMP and well separated from it.

Each of the radioactive materials obtained from the anion-exchange column was rechromatographed on Whatman no. 1 paper with the solvent systems described in the Methods section. The paper chromatography of AMP and GMP in solvent (8) showed that the radioactive peaks coincided well with the u.v.-absorbing spots as well as the authentic purine nucleotides co-chromatographed. Small amounts of radioactivity were also detected in the origin as well as in the solvent front. In contrast with the results for purine nucleotides, the radioactivities of peaks X and Y (Fig. 2) migrated into several fractions including the origin and the solvent front. Since peak Y merged into the CMP fractions on the anion-exchange column, it had a u.v.-absorbing spot corresponding to authentic CMP on the chromatograms, but no radioactivity was found in this spot. In contrast, the radioactivity of peak Z (Fig. 2) migrated to the solvent front. The radioactivities of peaks X, Y and Z are therefore probably due to contamination.

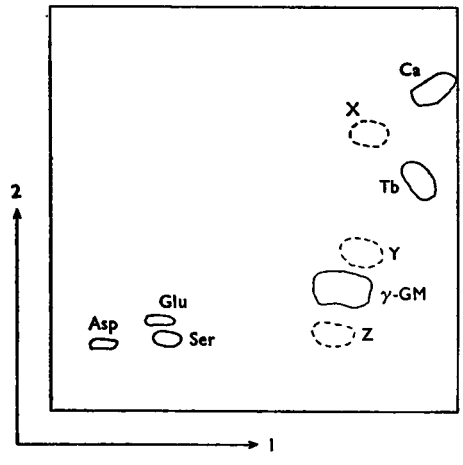


Fig. 1. *Tracing of a two-dimensional radioautogram of radioactive products of amino acids and methylated xanthines from tea shoot tips 6h after the absorption of* [^{14}C]*methylamine* (20μCi *in* 2ml *of solution*)

The 2M-NH_3 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. Abbreviations: γ-GM, γ-glutamylmethylamide; Ca, caffeine; Tb, theobromine; X, Y and Z, unknown substances.

Sequence of incorporation of radioactivity into products of methylamine metabolism in tea shoot tips

The time-course for incorporation of radioactivity from [^{14}C]methylamine into amino acids, theobromine, caffeine and the RNA purine nucleotides was investigated by feeding tea shoot tips with [^{14}C]-methylamine (5μCi in 0.5ml of solution) within 1h and incubating the tips in water for 3, 10, 30 and 90h. These times were chosen because of the known rates of incorporation of [^{14}C]methylamine into caffeine in tea shoot tips (T. Suzuki, unpublished work). Fig. 3 shows the time-course for the disappearance of radioactivity of [^{14}C]methylamine from tea shoots after the uptake of [^{14}C]methylamine. Radioactivity decreased rapidly during the first 10h incubation period, but a considerable amount of radioactivity remained in methylamine at 90h.

Labelling of amino acids, theobromine and caffeine in tea shoot tips is shown in Fig. 4. The radioactivity of serine reached a maximum within the first 10h incubation period, then decreased slowly. In contrast, the radioactivity of γ-glutamylmethylamide increased rapidly within the 3h incubation period and continued to increase slightly throughout the experimental period. The radioactivity of caffeine increased slowly and reached a maximum at 90h, whereas the radioactivity of theobromine reached a peak within the first 3h after the absorption of [^{14}C]methylamine, and then decreased slowly. The labelling of glutamate

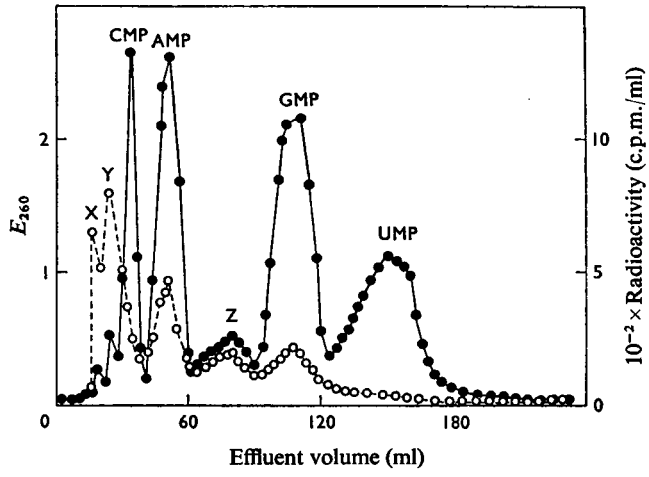


Fig. 2. *Ion-exchange chromatography of alkaline hydrolysate of RNA prepared from tea shoot tips (6g fresh wt.) labelled with [^{14}C]methylamine (20μCi) for 96h*

A Dowex 1 (X2; formate form) column (20cm×0.6cm), eluted with a gradient of 1–4M-formate, was used. ●, E_{260}; ○, radioactivity (c.p.m./ml). Radioactive peaks X, Y and Z were not identified.

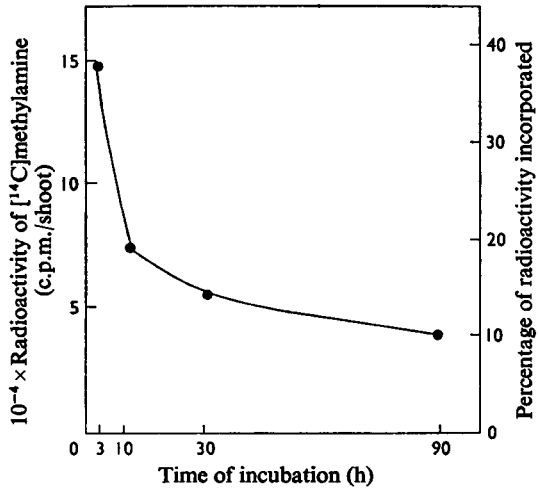


Fig. 3. *Time-course for the disappearance of the radioactivity of [^{14}C]methylamine from tea shoot tips after the uptake of [^{14}C]methylamine*

Groups of excised shoot tips (2.5–2.6g fresh wt.) were each fed with 5μCi of [^{14}C]methylamine (specific radioactivity 17mCi/mmol) within 1h, then were incubated in water in 50ml Erlenmeyer flasks for various periods. Methylamine was isolated on a column of Dowex 50W (X8; Na^+ form) as described in the text.

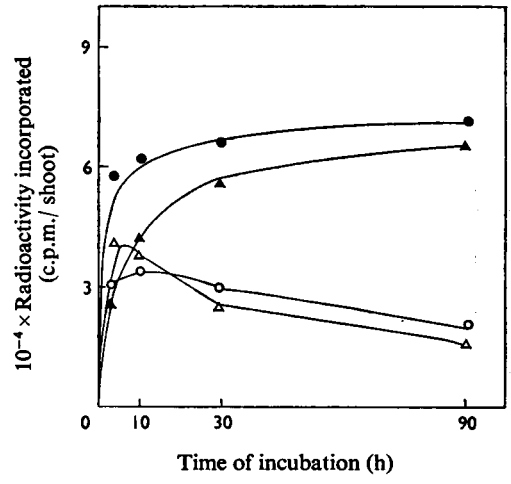


Fig. 4. *Incorporation of radioactivity from [^{14}C]methylamine into γ-glutamylmethylamide (●), serine (○), theobromine (△) and caffeine (▲) in tea shoot tips*

Groups of excised shoot tips were each fed with 5μCi of [^{14}C]methylamine within 1h, then were incubated in water for various periods. Other methods are described in the text.

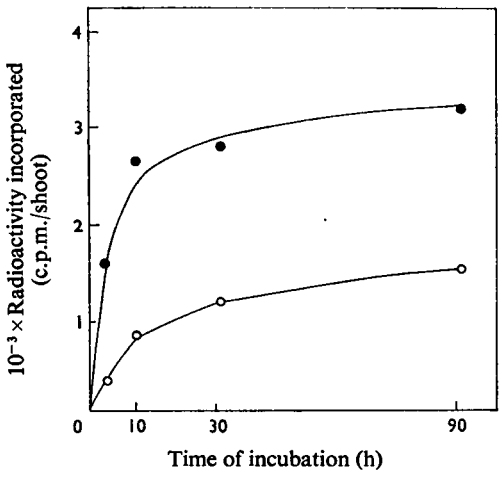


Fig. 5. *Incorporation of radioactivity from [^{14}C]-methylamine into the AMP (●) and GMP (○) of RNA in tea shoot tips*

Groups of excised shoot tips were each fed with 5 μCi of [^{14}C]methylamine within 1 h, then were incubated in water for various periods. Other methods are described in the text.

and aspartate was transient, disappearing 30 h after the feeding with [^{14}C]methylamine.

The pattern of incorporation of radioactivity from [^{14}C]methylamine into RNA purine nucleotides is shown in Fig. 5. Incorporation of radioactivity into AMP was faster and greater than that into GMP. In both nucleotides, however, the radioactivities continued to rise steadily during the 90 h experimental period.

Evidence for the formation of S-adenosylmethionine from methylamine in tea shoot tips

Although Preusser (1967) demonstrated that methylamine is a methyl donor in caffeine biosynthesis, its mode of transmethylation has not yet been clarified. It may be that methylamine is converted via *S*-adenosylmethionine into caffeine, because the formaldehyde released by the metabolism of methylamine may lead to the formation of *S*-adenosylmethionine (Cantoni, 1965). The importance of *S*-adenosylmethionine as a methyl donor in caffeine biosynthesis has been demonstrated by Suzuki (1972). In the present studies, tea shoot tips were fed with [^{14}C]methylamine (10 μCi) and the incorporation of ^{14}C into *S*-adenosylmethionine was examined after 10 h and 30 h experimental periods, during which time considerable synthesis of caffeine from [^{14}C]methylamine was shown to occur (Fig. 4). The acid-soluble extracts from tea shoot tips were applied to the Dowex 50W (Na^+ form) column. A single radioactive peak emerged during elution with 0.1 M-NaCl. This was identified as methylamine by the following evidence. First, the elution position coincided with that of an authentic sample. Secondly, paper chromatography in systems (1), (2) and (3) gave a radioactive spot with the same R_F value as authentic methylamine.

Radioactivity was also recovered in the 6 M-HCl fractions. Calculations showed that 0.6% and 0.2% of the total ^{14}C incorporated was recovered in the 6 M-HCl fractions of the 10 h and 30 h samples respectively. The radioactive material in the 6 M-HCl fractions was identified by co-chromatography on silica-gel plates with authentic *S*-adenosylmethionine in each of the four solvent systems described in the Methods section. In each solvent there was a complete overlap among the u.v.-absorbing spots, ninhydrin-positive spots and radioactive areas. In all systems, the R_F value of the radioactive material coincided with that of authentic *S*-adenosylmethionine.

γ-Glutamyl[^{14}C]methylamide-feeding experiments

Since the importance of γ-glutamylmethylamide as an intermediate in methylamine metabolism has been demonstrated by Kung & Wagner (1969), it was decided to examine the relationship it might have to methylamine metabolism in tea plants. Although its synthesis from methylamine and glutamate in tea plants (Konishi & Takahashi, 1966) as well as in a micro-organism, *Pseudomonas* MS (Kung & Wagner, 1969), has been demonstrated, the reverse reaction has not yet been confirmed. In the present studies, γ-glutamyl[^{14}C]methylamide (10 μCi) was given to tea shoot tips, and the products of γ-glutamylmethylamide metabolism were examined after a 10 h experimental period (2 h of absorption plus 8 h of incubation). Acid-soluble extracts were first applied to the Dowex 50W (Na^+ form) column. Methylamine was eluted from the column with 0.1 M-NaCl, and identified by paper chromatography in solvents (1) and (3). It was found that 2.9% of the total ^{14}C incorporated was converted into methylamine.

The 2 M-NH_3 eluate from the Amberlite IR-120 (H^+ form) resin was subjected to two-dimensional radioautography in solvents (4) and (5). Results were similar to those shown in Fig. 1. As in the [^{14}C]-methylamine-feeding experiments, serine, glutamate, aspartate, theobromine and caffeine were all important products of γ-glutamyl[^{14}C]methylamide metabolism.

DL-[3-^{14}C]*Serine-feeding experiments*

Attempts were also made to elucidate the incorporation of radioactivity from [^{14}C]methylamine into

glutamate and aspartate in tea shoot tips. Serine is a major product of methylamine metabolism. The formation of formaldehyde from methylamine and its subsequent entry into the C_1 pathway would result in the β-carbon of serine being labelled. Thus, in the present studies, DL-[3-^{14}C]serine (10μCi) was supplied to tea shoot tips, and the metabolic labelling of amino acids was examined after a 4h experimental period (2h of absorption plus 2h of incubation). Results indicated that glutamate, aspartate, glutamine, alanine, theobromine and caffeine were the major products of DL-[3-^{14}C]serine metabolism in tea shoot tips, but the largest amount of label was found in glutamate.

Discussion

Results of the present experiments clearly show that the tea plant possesses the ability to metabolize micromolar amounts of applied [^{14}C]methylamine (Table 1). This methylamine metabolism was associated with the major labelling of γ-glutamylmethylamide, serine, theobromine, caffeine and of the CO_2 evolved. RNA purine nucleotides and *S*-adenosylmethionine were also important products of methylamine metabolism in tea plants. These results are comparable with those observed in some microorganisms growing on methylamine as the sole carbon source, in that methylamine is oxidized via formaldehyde and formate to CO_2 (Eady & Large, 1968; Kung & Wagner, 1970).

The major conversion of methylamine into serine and the metabolic labelling of RNA purine nucleotides and *S*-adenosylmethionine suggest that in the tea plant the metabolism of methylamine proceeds via formaldehyde, and that its subsequent entry into the C_1 metabolic pool results in the labelling of all the cell constituents on the metabolic pathways of this compound. Formaldehyde can be produced from methylamine by the action of amine oxidase, which occurs widely in micro-organisms, animals and higher plants (Hill & Mann, 1968; Kapeller-Adler, 1971). It has been shown that the purified amine oxidase of pea seedlings readily oxidizes monoamines, diamines and polyamines (Hill & Mann, 1964).

The synthesis of serine from formaldehyde is probably catalysed by the enzyme serine hydroxymethyltransferase (EC 2.1.2.1). Experiments with plant tissue extracts have shown that serine hydroxymethyltransferase can utilize formaldehyde in a reaction dependent on tetrahydrofolate and glycine (Cossins & Sinha, 1966; Shah & Cossins, 1970*a*). The rapid conversion of glycine into serine in tea plants has been demonstrated by Roberts & Sanderson (1966).

The formation of γ-glutamylmethylamide from methylamine is of particular interest because of its possible importance in the metabolism of methylamine as demonstrated by Kung & Wagner (1969). Konishi *et al.* (1972*a*,*b*) established that the *N*-methyl carbon of γ-glutamylmethylamide is converted into caffeine in tea seedlings during growth.

In the present studies, after feeding with [^{14}C]-methylamine the radioactivity was rapidly incorporated into γ-glutamylmethylamide during the first 3h and remained fairly constant during later time-intervals, whereas the radioactivity of serine reached a maximum within the first 10h, then decreased slowly (Fig. 4). This is explicable if γ-glutamylmethylamide is directly formed from methylamine and glutamate, as suggested by Konishi & Takahashi (1966), whereas radioactivity is incorporated into serine as a result of the oxidation of methylamine. In contrast, in the γ-glutamyl[^{14}C]methylamide-feeding experiments, methylamine was the product of γ-glutamylmethylamide metabolism, indicating that the reaction leading to γ-glutamylmethylamide formation is reversible. Further, as in the [^{14}C]methylamine-feeding experiments, serine, glutamate, aspartate, theobromine and caffeine were all obtained as important products of γ-glutamyl[^{14}C]methylamide metabolism. It is likely, therefore, that the *N*-methyl carbon of γ-glutamylmethylamide is converted into caffeine via methylamine and formaldehyde through the C_1 pathway in tea plants.

From the conclusions of Konishi *et al.* (1969, 1972*a*) and the present results, γ-glutamylmethylamide appears to play a role in the reversible storage or transport of methylamine in its non-toxic form in tea plants. [^{14}C]Methylamine-feeding experiments with germinating seeds and excised root tips of tea plants have shown that almost all the radioactivity in the 80%-ethanol extract is found in γ-glutamylmethylamide (Konishi *et al.*, 1969). It is probable, therefore, that γ-glutamylmethylamide is mainly formed in roots and is transported to leaves, where it is converted into caffeine via methylamine. These observations on γ-glutamylmethylamide metabolism in tea plants are comparable with those of Kito *et al.* (1966, 1968) for the metabolism of theanine, the γ-glutamylethylamide analogue of glutamine, in that the *N*-ethyl carbon of theanine is a precursor of catechins, important components of tea leaves. However, in both cases the biogenesis of amines remains to be solved.

Besides the labelling of two major products (γ-glutamylmethylamide and serine), tea shoot tips produced two other major labelled products (theobromine and caffeine), after the tips had been fed with [^{14}C]methylamine (Fig. 1). The pattern of incorporation of ^{14}C into theobromine and caffeine (Fig. 4) indicated that initially theobromine was the more rapidly labelled product, whereas much more radioactivity was found in caffeine after 10h. Similar observations were made by Ogutuga & Northcote (1970) for caffeine biosynthesis in tea plant callus

tissue fed with L-[*Me*-^{14}C]methionine. The labelling of theobromine and caffeine was also shown to occur in tea shoot tips as a result of feeding with DL-[3-^{14}C]serine. All these facts suggest that theobromine is the immediate precursor of caffeine.

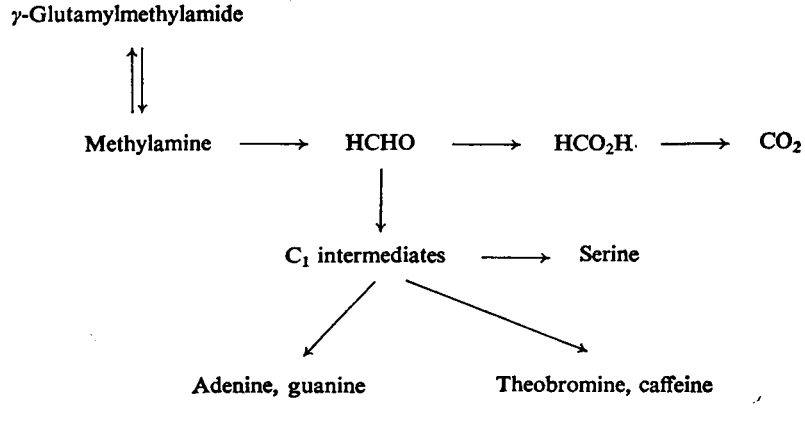
Methylamine has been shown to be a methyl donor in caffeine biosynthesis (Preusser & Serenkov, 1963; Preusser, 1967). The present results indicate that the formaldehyde released by the metabolism of methylamine enters the C_1 metabolic pool, resulting in the formation of *S*-adenosylmethionine. It is conceivable, therefore, that methylamine is converted via methionine and *S*-adenosylmethionine into caffeine. There is now considerable evidence that higher plants synthesize methionine by a reaction dependent on homocysteine and 5-methyltetrahydrofolate (Burton & Sakami, 1969; Shah & Cossins, 1970*b*; Dodd & Cossins, 1970). The formation of *S*-adenosylmethionine from methionine in tea plants has been shown to occur (Suzuki, 1972). Finally, the involvement of *S*-adenosylmethionine in caffeine biosynthesis has been demonstrated by Suzuki (1972).

In contrast with the results with *S*-adenosylmethionine, no labelling of methionine was detected (Fig. 1). This was probably due to the rapid turnover rate and small pool-size of methionine in tea plants. Paper chromatography of free amino acids from tea shoots showed that the spot corresponding to authentic methionine was ninhydrin-negative. Further, L-[*Me*-^{14}C]methionine-feeding experiments with tea shoots (T. Suzuki, unpublished work) have shown that almost all the L-[*Me*-^{14}C]methionine supplied is metabolized within a 3h experimental period (2h of absorption plus 1h of incubation), and that this utilization results in heavy labelling of theobromine, caffeine and an unknown compound and in slight labelling of serine, glutamate and aspartate.

In addition to the labelling of serine and *S*-adenosylmethionine by C_1 compounds, tea shoot tips fed with [^{14}C]methylamine produced RNA purine nucleotides as other important products of C_1 metabolism (Fig. 2). It is now well established that C-2 and C-8 of the purines adenine and guanine, but none of the ring carbon atoms of the pyrimidines, are derived from the C_1 pool, and that C-6 of purines and C-2 of the pyrimidines are derived from CO_2 (Hartman, 1970). C-4 and C-5 of purines are derived from glycine. Since glycine and RNA pyrimidine nucleotides were not the products of methylamine metabolism in tea plants (Figs. 1 and 2), it can be assumed that the labelling of the RNA purine nucleotides was from the C_1 pool. This labelling of RNA purine nucleotides is therefore consistent with the suggestion that methylamine can serve as a C_1 precursor.

Labelling of glutamate and aspartate occurred in tea shoot tips as a result of feeding them with adequate amounts of [^{14}C]methylamine (Fig. 1). Administration of less [^{14}C]methylamine to tea shoot tips was not sufficient to detect the labelling (Konishi *et al.*, 1969). Pyruvate might be produced from serine by the action of serine dehydratase (Greenberg, 1962). In plants, Hill & Rogers (1972) reported serine dehydratase activity in French beans. The present studies clearly show the incorporation of [^{14}C]-methylamine into organic acids (Table 1). Further, glutamate, aspartate, glutamine and alanine were shown to be the major products of DL-[3-^{14}C]serine metabolism in tea shoot tips. Thus labelling of glutamate and aspartate could occur if the labelled serine produced from [^{14}C]methylamine was metabolized via the tricarboxylic acid cycle.

It is also likely that a considerable amount of radioactivity from [^{14}C]methylamine was converted via formaldehyde into the evolved CO_2. The value for



Scheme. 1. *Metabolism of methylamine in the tea plant*

incorporation from [^{14}C]methylamine into either the residue or CO_2 is 36% of the ^{14}C incorporated (Table 1). DNA and proteins might also be labelled as a result of [^{14}C]methylamine metabolism in tea plants, but, from results of the incorporation of [^{14}C]methylamine into RNA and amino acids, the amount incorporated into DNA and proteins must be small. CO_2 presumably contains most of the ^{14}C incorporated into 'other compounds' referred to in Table 1. The conversion of methylamine into CO_2 in tea shoot tips probably proceeds in a manner similar to that known for other organisms (Eady & Large, 1968; Kung & Wagner, 1970). In plants, Davies (1968) has shown that swede discs can readily metabolize formaldehyde to CO_2. Further, enzyme systems responsible for the oxidation of methanol to CO_2 in higher plants have been demonstrated by Cossins (1964). Hence it is possible that in tea plants CO_2 is formed from formaldehyde, itself produced from [^{14}C]methylamine by the action of the aldehyde oxidase or dehydrogenase followed by the action of formate dehydrogenase (Davison, 1951; Cossins & Sinha, 1965).

From the present studies it appears that the metabolism of methylamine in tea plants occurs as summarized in Scheme 1. Metabolism of [^{14}C]-methylamine by these pathways would lead to the distribution of the radioactivity among the major isolated products. In addition, Preusser (1967) has shown that the caffeine content of coffee plants also increases on feeding with methylamine, and Scheme 1 (described for tea plants) presumably describes the events in coffee plants, with the exception of the conversion of methylamine into γ-glutamylmethylamide.

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