

DISTRIBUTION AND BIOSYNTHESIS OF CAFFEINE IN PLANTS

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1. ABSTRACT

Methylxanthines and methyluric acids are secondary metabolites derived from purine nucleotides and are known collectively as purine alkaloids. The present review begins by summarizing the distribution of these compounds in the plant kingdom, and then provides an up-to-date account of the biosynthesis of purine alkaloids. The diversity of biosynthesis and accumulation between species and between tissues of different age is considered. We also discuss the physiological function of these purine alkaloids in plants and the biotechnology for creating caffeine-free plants.

2. INTRODUCTION

Methylxanthines and methyluric acids are derivatives of purine nucleotides. These compounds are generally called purine alkaloids. Caffeine (1, 3, 7-trimethylxanthine) is synthesized in some groups of higher plants, such as tea (*Camellia sinensis*) and coffee (*Coffea arabica*, *Coffea canephora*). The pharmacological effects

of caffeine, including stimulation of the central nervous system, have been extensively investigated. Modern experimental studies of the biosynthetic pathway of caffeine were initiated by several investigators in the 1970s, including Suzuki, one of the present authors (1, 2). Since then, data supporting the pathway, xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine have accumulated. However, several different pathways for caffeine and theobromine biosynthesis were proposed in the 1990s (3, 4). The original pathway proposed by Suzuki and Takahashi (1, 2) was confirmed using modern instruments as a major pathway of caffeine biosynthesis by the group led by the other author of the present article: Ashihara *et al.* (5). An *N*-methyltransferase enzyme that catalyzes the conversion of 7-methylxanthine to caffeine has been demonstrated in tea-leaf extracts by Suzuki and Takahashi (2). Twenty-five years later, this enzyme was highly purified to apparent homogeneity by Kato *et al.* (6) and named caffeine synthase. Using the sequence of *N*-terminal amino

Table 1. Distribution of purine alkaloids in plants. Major purine alkaloids shown are examples observed in mature leaves or seeds. For details, see text and Refs 13-15

Latin name	Common name	Major alkaloid
<i>Coffea arabica</i>	Arabica coffee	caffeine
<i>Coffea canephora</i>	Robusta coffee	caffeine
<i>Coffea liberica</i>		theacrine, libertine
<i>Coffea dewevrei</i>		theacrine, libertine
<i>Camellia sinensis</i>	tea	caffeine
<i>Camellia assamica</i>	Assam tea	caffeine
<i>Camellia assamica</i> <i>var Kucha</i>	kucha	theacrine
<i>Camellia taliensis</i>		caffeine
<i>Camellia</i> <i>irrawadiensis</i>		theobromine
<i>Camellia ptilophylla</i>	cocoa tea	theobromine
<i>Theobroma cacao</i>	cacao (cocoa)	theobromine
<i>Theobroma gradiflorum</i>	cupu	liberine
<i>Paullinia cupana</i>	guarana	caffeine
<i>Cola sp.</i>		caffeine
<i>Cirus sp.</i>		caffeine

acids of this caffeine synthase, full-length cDNA of caffeine synthase was successfully cloned (7). Genes of *N*-methyltransferases that catalyze all three methylation steps have now been cloned in coffee plants by two independent Japanese groups (8-12). The cloning of caffeine biosynthesis genes makes genetic engineering possible to produce naturally decaffeinated tea and coffee.

Review articles of purine alkaloid metabolism have already been published (13-16). This review will outline the biosynthesis and accumulation of caffeine and related purine alkaloids in higher plants, including recent results reported in 2000-2004. The related topics, "catabolism of purine alkaloids" and "detailed properties of *N*-methyltransferases for caffeine biosynthesis" will be reviewed by Mazzafera and by Kato and Mizuno, in the following chapters.

3. STRUCTURE AND PROPERTIES OF PURINE ALKALOIDS FOUND IN NATURE

Purine alkaloids consist of methylxanthines and methyluric acids. The most abundant purine alkaloid in nature is caffeine (1, 3, 7-trimethylxanthine), followed by theobromine (3, 7-dimethylxanthine). Small amounts of theophylline (1, 3-dimethylxanthine), paraxanthine (1, 7-dimethylxanthine), 1-methylxanthine, 3-methylxanthine, 7-methylxanthine and 7-methylxanthosine are also found. These minor components may be intermediates of caffeine biosynthesis and/or catabolism. The melting points of caffeine, theobromine and theophylline are respectively 238, 357 and 270°C (Merck Index), and their sublimation points are 178, 290 and 269 °C (Merck Index, 17). Caffeine dissolves well in boiling water, but at room temperature, chloroform is a better solvent. Theobromine is less soluble in water than caffeine, but it dissolves readily in aqueous acids and alkalis.

Chlorogenic acid forms a 1:1 complex with caffeine. Details of chemical and physical properties of purine alkaloids are given by Tarka and Hurst (18).

4. DISTRIBUTION OF PURINE ALKALOIDS

Compared with other alkaloids, such as nicotine, morphine and strychnine, purine alkaloids are distributed widely throughout the plant kingdom. Caffeine has been found in 13 orders of the plant kingdom. Most caffeine-containing plants are members of the dicotyledoneae, although *Scilla maritima* belongs to the monocotyledoneae. In some species the main purine alkaloid is theobromine or methyluric acids including theacrine (1, 3, 7, 9-tetramethyluric acid), rather than caffeine (14, 16) (Table 1).

4.1. Coffee and related *Coffea* plants

The caffeine content of seeds of different *Coffea* species varies from 0.4 to 2.4% dry weight (19). Most cultivars of *Coffea arabica* contain ca. 1% caffeine. There are some *Coffea* species containing high levels of caffeine. These are *Coffea canephora* (1.7%), *Coffea dewevrei* (1.2%) and *Coffea liberica* (1.4%). The low caffeine *Coffea* species are *Coffea eugenioides* (0.4%), *Coffea salvatrix* (0.7%) and *Coffea racemosa* (0.8%). During the development of *Coffea arabica* fruits, the caffeine content of the pericarp falls from 2% to 0.2% d.w., but remains constant in the seeds at 1% (20). Young expanding leaves of *Coffea arabica* plants also contain theobromine, though in lower levels than caffeine (21, 22). In 6-month-old *Coffea arabica* seedlings, caffeine was distributed mainly in leaves and cotyledons, at concentrations varying from 0.8-1.9% dry wt. Essentially no caffeine was detected in roots or in the older brown parts of shoots (23). Mature leaves of *Coffea liberica*, *Coffea dewevrei* and *Coffea abeokutae* contain the methyluric acids, theacrine (1, 3, 7, 9-tetramethyluric acid), liberine (*O* (2), 1, 9-trimethyluric acid) and methyl-liberine (*O* (2), 1, 7, 9-tetramethyluric acid (24, 25).

4.2. Tea and related *Camellia* plants

Nagata and Sakai (26) examined the distribution of caffeine in 23 species of the genus *Camellia*. The caffeine contents of young leaves of first flush shoots of *Camellia sinensis* var. *sinensis*, *Camellia sinensis* var. *assamica*, and *Camellia taliensis* were 2-3%, and its caffeine in *Camellia kissi* was less than 0.02%. Theobromine was the predominant purine alkaloid in young leaves of *Camellia ptilophylla* (5.0-6.8%)(27) and *Camellia irrawadiensis* (<0.8%)(28). Theacrine (1, 3, 7, 9-tetramethyluric acid) and caffeine were the major purine alkaloids in the leaves of an unusual Chinese tea known as kucha (*Camellia assamica* var. kucha). Endogenous levels of theacrine and caffeine in expanding buds and young leaves were ca. 2.8 and 0.6-2.7% of the dry wt, respectively, but the concentrations were lower in mature leaves (29).

The stamens and petals of several species of *Camellia* plants contain caffeine and/or theobromine (30); and the species-to-species pattern of purine alkaloid distribution is very similar to that found in young leaves

(31). The distribution of purine alkaloids in seeds and seedlings of *Camellia sinensis* has been examined by Ashihara and Kubota (32). In seeds, most of the caffeine is located in the seed coat, and only a trace was detected in the embryo. More than 99% of caffeine in 4-month-old seedlings was in the leaves, with minor pools in stems, roots, and cotyledons.

4.3. Cacao and related *Theobroma* and *Herrania* plants

Theobromine is the dominant purine alkaloid in seeds of cacao (*Theobroma cacao*). Changes in the levels of theobromine and caffeine during the growth of cacao beans have been monitored by Senanayake and Wijesekera (33). The cotyledons of mature beans contain theobromine (2.2-2.7% dry weight) and caffeine (0.6-0.8%); lower levels, theobromine (0.6-0.7%) and caffeine (0.5-0.6%), are found in the shell of the seed. Zheng *et al.* (34) investigated the purine alkaloid content of *Theobroma cacao* fruits at different growth stages: Stage A (young small fruit, fresh weight *ca.* 2 g); stage B (middle size fruit, fresh weight *ca.* 100 g) and stage C (large size, fresh weight *ca.* 500 g). The major purine alkaloid in stage A fruits (mainly pericarp) is theobromine, followed by caffeine. The theobromine content of the pericarp decreased sharply with the age of tissue, and the caffeine content decreased gradually. Large amounts of theobromine (0.4% fresh weight) accumulate in seeds (mainly cotyledons) of stage C fruits. Theobromine was also found in the seed coat and placenta. Koyama *et al.* (35) examined the purine alkaloid content of cacao leaves at various ages: young small leaves (stage I), developing intermediate size leaves (stage II), fully developed leaves (stage III) from flush shoots, and aged leaves (stage IV) from 1-year-old shoots. The major purine alkaloid in stage I leaves is theobromine (4.5 $\mu\text{mol/g}$ fresh weight), followed by caffeine (0.75 $\mu\text{mol/g}$ fresh weight). More than 75% of purine alkaloids disappeared with subsequent leaf development (stages II to IV).

Hammerstone *et al.* (36) found that the seeds of 11 *Theobroma* species and those of 9 *Herrania* species contained tetramethyluric acid (theacrine) as the principal purine alkaloid. Baumann and Wanner (37) reported that cupu (*Theobroma grandiflorum*), contained 0.25 % liberine in cotyledons and 0.08 % in nut shells.

4.4. Other plants

Purine alkaloids are also present in the leaves of maté (*Ilex paraguariensis*). Young maté leaves contain 0.8-0.9% caffeine, 0.08-0.16% theobromine and <0.02% theophylline (38). Much lower levels are found in mature leaves, and trace amounts of caffeine and theobromine are found in fruits, bark, and wood of maté (38). Caffeine and theobromine were found recently in leaf epicuticular waxes of maté (39).

The distribution of purine alkaloids in the various parts of the fruit and seeds of guaraná (*Paullinia cupana*) has been investigated by Baumann *et al.* (40). In seeds, caffeine is the major methylxanthine, with the cotyledons containing 4.3% and the testa 1.6%. Both tissues also contained traces of theobromine (<0.2%) and theophylline (<0.01%). The pericarp contains lower levels of purine

alkaloids, but theobromine (0.2%) was the main component together with trace amounts of caffeine (0.02%) and theophylline (0.001%). Weckerle *et al.* (41) screened 34 species of *Paullinia* and related genera for purine alkaloids; only three species, *P. cupana*, *P. yoco*, and *P. pachycarpa*, were positive. The distribution of purine alkaloids in *P. pachycarpa* was found to be restricted to theobromine, in the stem, leaves, and flowers.

Significant amounts of caffeine and theophylline were recently detected in flowers of various *Citrus* species (42). Most caffeine was found in the androecium. In the anther these purine alkaloids reach a concentration of 0.9% dry wt. Similar concentrations of alkaloids occur in pollen. Nectar also contains small amount of alkaloids.

5. THE "CORE PATHWAY" OF CAFFEINE BIOSYNTHESIS: CONVERSION OF XANTHOSINE TO CAFFEINE

Exogenously supplied ^{14}C -labeled purine bases and nucleosides have been used experimentally as precursors for caffeine biosynthesis (e.g., 5, 22, 29-32, 35, 43-45). Those studies indicate that the purine skeleton of caffeine is derived from purine nucleotides. Pulse-chase experiments with [8- ^{14}C]xanthosine and young *Coffea arabica* leaves found that 7-methylxanthine and theobromine were both labeled during a 6-h pulse, but the radioactivity became associated almost exclusively with caffeine after an 18-h chase (5). These ^{14}C -tracer studies using segments of plant tissues and the substrate specificity of enzymes for caffeine biosynthesis strongly suggest that the major pathway from xanthosine to caffeine is xanthosine \rightarrow 7-methylxanthosine \rightarrow 7-methylxanthine \rightarrow theobromine \rightarrow caffeine (Figure 1). As well as this main pathway, some minor routes, such as 7-methylxanthine \rightarrow paraxanthine \rightarrow caffeine, and xanthine \rightarrow 3-methylxanthine \rightarrow theobromine \rightarrow caffeine, may be functioning (5, 46). These minor pathways may be a result of the broad methyl acceptor specificity of the *N*-methyltransferase(s), which is discussed elsewhere (16).

5.1. Production of 7-methylxanthosine

The first methylation enzyme, xanthosine *N*-methyltransferase, to catalyze the formation of 7-methylxanthosine from xanthosine (Step 1 in Figure 1), was originally demonstrated in tea leaf extracts by Negishi *et al.* (47). The presence of this enzyme was confirmed by other investigators (46, 48). Baumann and co-workers suspected that 7-methylxanthosine was not an intermediate of caffeine biosynthesis in coffee, because it was not detected in extracts from cultured coffee cells even when caffeine biosynthesis was stimulated by adenine and ethephon (49). Using cell-free extracts of coffee leaves containing the nucleotidase inhibitor Na_2MO_4 , Schulthess *et al.* (50) also demonstrated that both xanthosine and XMP were utilized as a methyl acceptor of SAM. Based on these observations, they proposed a new pathway XMP \rightarrow 7-methyl-XMP \rightarrow 7-methylxanthosine \rightarrow 7-methylxanthine. However, recent molecular studies do not support this XMP pathway (10, 12).

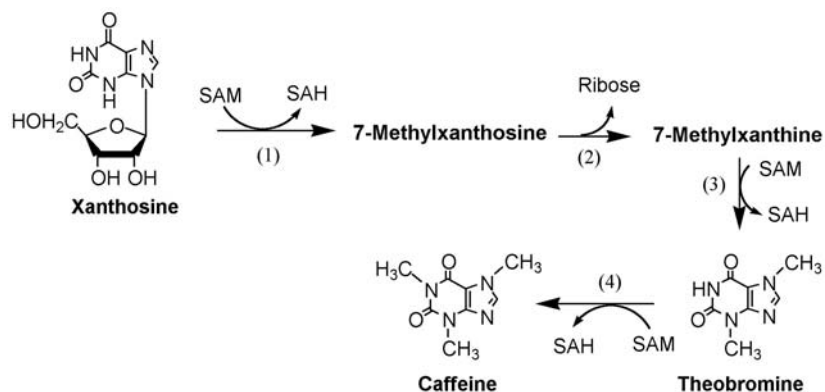


Figure 1. The “core pathway” of caffeine biosynthesis in plants. Enzymes: (1) 7-methylxanthosine synthase (xanthosine *N*-methyltransferase, EC 2.1.1.-), (2) *N*-methylxanthine nucleosidase (EC3.1.3.-), (3) theobromine synthase (7-methylxanthine *N*-methyltransferase, EC 2.1.1.-) and/or caffeine synthase (7-methylxanthine and theobromine *N*-methyltransferase, EC 2.1.1.-), (4) caffeine synthase (7-methylxanthine and theobromine *N*-methyltransferase, EC 2.1.1.-). EC numbers have not yet been given for all enzymes of this pathway.

Genes encoding the enzyme for the first methylation step of caffeine biosynthesis have recently been cloned. Mizuno *et al.* (10) identified and characterized the gene encoding the first *N*-methyltransferase. The full-length cDNA was translated with an *Escherichia coli* expression system and the resultant recombinant protein was purified using a Ni-NTA column. This protein, named 7-methylxanthosine synthase, shows xanthosine *N*-methyltransferase activity, but XMP could not be used as a substrate. The gene encoding 7-methylxanthosine synthase was also cloned from immature coffee fruits by Uefuji *et al.* (12). The recombinant protein shows xanthosine specific activity. The available data therefore suggest that xanthosine, but not XMP, is the methyl acceptor in coffee plants.

5.2. Hydrolysis of 7-methylxanthosine to 7-methylxanthine

Conversion of 7-methylxanthosine to 7-methylxanthine appears to be catalyzed by a specific *N*-Methyl nucleosidase (Step 2 in Figure 1). This enzyme has been partially purified from tea leaves by Negishi *et al.* (51). The substrate specificity of this enzyme was rather broad, and high activity was found with several monomethyl purine nucleosides. The estimated molecular mass was 55 kD and the optimum pH was 8.0-8.5. *N*-Methyl nucleosidase was readily separated from adenosine nucleosidase by DEAE-cellulose chromatography.

5.3. Production of caffeine from 7-methylxanthine via theobromine

The conversion of 7-methylxanthine to caffeine includes two *N*-methyltransfer reactions (Steps 3 and 4 in Figure 1). Activities of SAM: 7-methylxanthine *N*-methyltransferase and SAM: theobromine *N*-methyltransferase, which respectively catalyze the second and third methylation steps in the main pathway, were first demonstrated in crude tea leaf extracts by Suzuki and Takahashi (2), who showed that the two enzymes have identical pH optima and are affected similarly by metal ions and inhibitors. Since then, *N*-methyltransferase activity has been detected in cell-free extracts prepared from immature fruits (52) and cell-suspension cultures of *Coffea*

arabica (53). Fujimori *et al.* (48) confirmed the activities of these *N*-methyltransferases in tea leaf extracts, and found that they were highest in very young expanding leaves but absent in fully developed leaves.

Purification of *N*-methyltransferase(s) has been attempted by several investigators. Mazzafera *et al.* (54) reported the purification of a *N*-methyltransferase from fruits and leaves of *Coffea arabica*, which possessed 7-methylxanthine and theobromine *N*-methyltransferase activity. Kato *et al.* (46) partially purified *N*-methyltransferase from tea leaves by ion-exchange and gel-filtration chromatography. Mosli Waldhauser *et al.* (55, 56) purified *N*-methyltransferases from coffee leaves up to 39-fold using ion-exchange chromatography and chromatofocusing. However, purification was very difficult, as a result of the instability of enzyme in extract, so that no homogeneity preparation of *N*-methyltransferase was obtained until 1999. A *N*-methyltransferase from young tea leaves was purified 520-fold to apparent homogeneity by Kato *et al.* (6) with several purification steps: ammonium sulfate fractionation and subsequent chromatography steps with hydroxyapatite, Shodex IEC QA anion-exchange, adenosine-agarose and Superdex 200 gel-filtration chromatography. The protein exhibited broad substrate specificity and catalyzed the second and third methylations in the caffeine biosynthetic pathway, namely the conversion of 7-methylxanthine to caffeine via theobromine (Figure 1). The single *N*-methyltransferase so obtained was referred to by authors (6) as caffeine synthase (CS). By using the technique of 3'-rapid amplification of cDNA ends with degenerate gene-specific primers based on the *N*-terminal residues of purified tea CS, a 1.31 kb sequence of cDNA has been obtained (7). The 5'-untranslated sequence of the cDNA fragment was isolated by 5'-rapid amplification of the cDNA ends. The total length of the isolated cDNA, termed *TCSI*, is 1438 bp and encodes a protein of 369 amino acids. The substrate specificity of the recombinant enzyme expressed in *E. coli* is similar to that of purified CS from young tea leaves. The recombinant enzyme catalyzed mainly *N*-1-

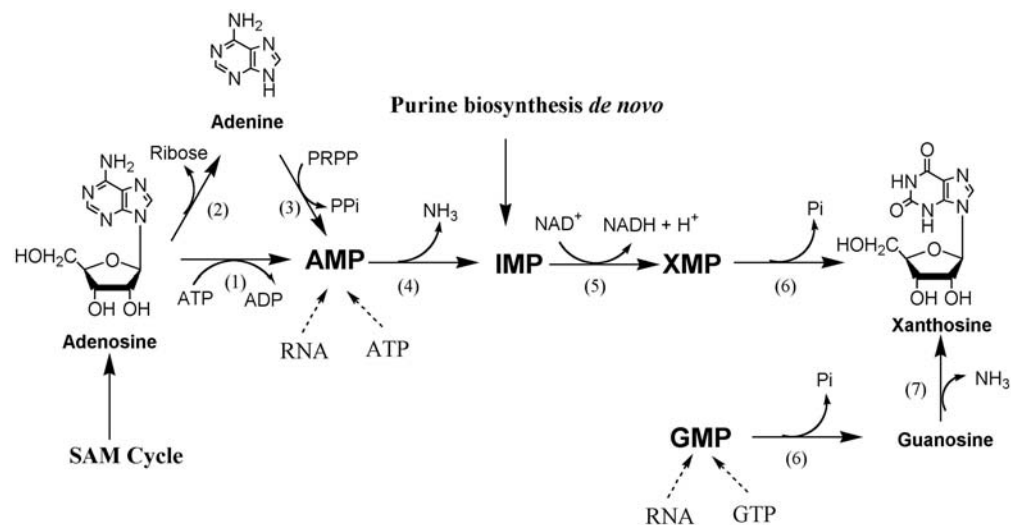


Figure 2. The “provider pathways” for xanthosine synthesis in purine alkaloid forming plants. Enzymes: (1) adenosine kinase (EC 2.7.1.20), (2) adenosine nucleosidase (EC 3.2.2.7), (3)adenine phosphoribosyltransferase (2.4.2.7), (4) (5) AMP deaminase (EC 3.5.4.6), (5) IMP dehydrogenase (1.1.1.205), (6) 5'-nucleotidase (EC 3.1.3.5), (7) guanosine deaminase (EC 3.5.4.15).

methylation and *N*-3-methylation of mono- and dimethylxanthines. No 7-*N*-methylation activity is observed when xanthosine or XMP is used as the methyl acceptor. These results provide convincing evidence that *TCSI* encodes CS. Recently, Mizuno *et al.* (9) reported the isolation of a bifunctional coffee caffeine synthase (*CCSI*) clone from coffee endosperm. The predicted amino acid sequences of *CCSI* are about 40% similar to those of tea caffeine synthase (*TCSI*). Like tea *TCSI*, the recombinant enzyme *CCSI* has dual methylation activity.

As well as the dual functional *N*-methyltransferases, the genes encoding a theobromine synthase (SAM: 7-methylxanthine *N*-methyltransferase) have also been cloned from coffee plants. Mizuno *et al.* (8) found several similar genes in coffee plants. Two of the coffee genes, *CTS1* and *CTS2*, were expressed in *E. coli*. The substrate specificity of the recombinant coffee enzymes was much more restricted than that of recombinant CS because they use only 7-methylxanthine as a methyl acceptor, converting it to theobromine. The coffee *N*-3-methyltransferases are therefore, referred to as theobromine synthases (8). Independently, Ogawa *et al.* (11) cloned similar genes from coffee leaves. Upon expression in *E. coli*, one of these genes was found to encode a protein possessing *N*-3-methylation activity. Although native enzymes have not yet been obtained from plant materials, theobromine synthase is certainly present in coffee plants and may participate in caffeine biosynthesis.

5.4. Regulation of the “core pathway”

The rate of caffeine biosynthesis appears to be regulated primarily by the induction and repression (so-called “coarse control”) of 7-methylxanthine synthase and caffeine synthase, or in some cases theobromine synthase. Kato (57) compared the expression of tea caffeine synthase in tea leaves of different ages. The Northern blot analysis using a 527 bp cDNA fragment of *TCSI* found a mRNA

signal in caffeine synthesizing leaves, as predicted from tracer experiments using ^{14}C -precursors (48); and the strongest signal was found in young leaves, in which the caffeine synthase activity in tea extracts was high (48). Regulation of the transcription of caffeine-related enzyme genes is therefore a highly plausible mechanism.

The regulation of these *N*-methyltransferase enzyme activities by the SAM/SAH ratio (“fine control”) may also be involved in regulating the ‘core pathway’. Upon donation of the methyl group to acceptor molecules, SAM is converted to SAH. Since SAH is a potent inhibitor of methyltransferases including caffeine synthase (6), removal of SAH by SAH hydrolase is essential for the continued operation of the caffeine biosynthesis *N*-methyltransferases. The SAH hydrolase reaction is reversible, and the equilibrium lies heavily in favour of SAH synthesis from adenosine and homocysteine. Removal of adenosine and homocysteine is therefore essential for the effective hydrolysis of SAH; this is achieved by metabolism of homocysteine to methionine, which is further converted to SAM to complete the SAM regeneration cycle (58). Furthermore, the availability of purine precursors originating from *de novo* purine biosynthesis and purine salvage pathways may regulate the biosynthesis of caffeine. Feedback control of purine alkaloids by end products has not previously been discovered.

6. THE “PROVIDER PATHWAYS” FOR XANTHOSINE

Xanthosine is a purine nucleoside that is produced by degradation of purine nucleotides. There are some purine pools in plant cells, specifically free adenine nucleotides, guanine nucleotides, SAM and nucleic acids. Theoretically, therefore, xanthosine is formed from these compounds (Figure 2). In the early 1970s it was believed that a major source of caffeine was methylated nucleic

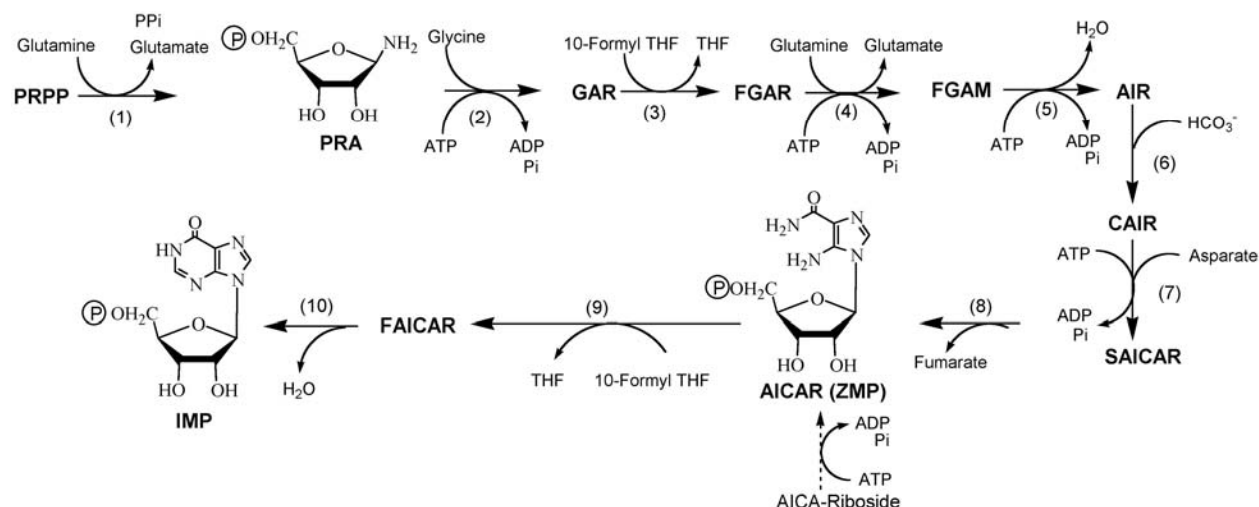


Figure 3. *De novo* biosynthetic pathway of IMP in plants. Metabolites: PRPP, 5-phosphoribosyl-1-pyrophosphate; PRA, 5-phosphoribosyl amine; GAR, glycineamide ribonucleotide; FGAR, formylglycineamide ribonucleotide; FGAM, formyl glycine amidine ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; CAIR, 5-aminoimidazole 4-carboxylate ribonucleotide; SAICAR, 5-aminoimidazole-4-*N*-succinocarboxamide ribonucleotide; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, 5-formamidoimidazole-4-carboxamide ribonucleotide. Enzymes: (1) Amido phosphoribosyltransferase (EC 2.4.2.14); (2) GAR synthetase (EC 6.3.4.13); (3) GAR formyl transferase (EC 2.1.2.2); (4) FGAM synthetase (EC 6.3.5.3); (5) AIR synthetase (EC 6.3.3.1); (6) AIR carboxylase (EC 4.1.1.21); (7) SAICAR synthetase (EC 6.3.2.6); (8) adenylosuccinate lyase (EC 4.3.2.2); (9) ZMP formyltransferase (EC 2.1.2.3); (10) IMP cyclohydrolase (3.5.4.10). N.B. The abbreviation AICAR is often used for both 5-aminoimidazole-4-carboxamide ribonucleotide and 5-aminoimidazole-4-carboxamide ribonucleoside. In this review, AICAR is used for the ribonucleotide. The names of the intermediates and enzymes used in this review are not based on the recommendations of the nomenclature committee of the International Union of Biochemistry and Molecular Biology, because the more traditional terminology is still well established in this field.

acids (59). However, recent research does not support this hypothesis, and free purine nucleotides are the major resources for xanthosine formation.

6.1. *De novo* purine biosynthesis

Anderson and Gibbs (60) and Proiser and Serenkov (61) first demonstrated that the carbon atoms of purine skeletons of caffeine, purine nucleotides, and nucleic acids derive from the same precursors. However, there is little information on whether caffeine and other purine alkaloids are formed directly from an intermediate of the *de novo* pathway of purine biosynthesis. Ito and Ashihara (62) reported that young tea leaves incorporated the ¹⁵N atom from [¹⁵N]glycine into theobromine and caffeine, and that incorporation was markedly reduced by azaserine and aminopterin, which are known inhibitors of purine biosynthesis *de novo*. Furthermore, the radioactivity from [2-¹⁴C]AICA-riboside, a precursor of an intermediate of the pathway, was also incorporated into theobromine and caffeine (Figure 3). To determine whether purine alkaloids are produced from an intermediate (IMP) of the *de novo* pathway or from the adenine nucleotide pools, the effect was studied of coformycin on the biosynthesis of purine alkaloids from [2-¹⁴C]AICA-riboside (62). Inhibition of the conversion of AMP to IMP by coformycin, which is an inhibitor of AMP deaminase, did not significantly alter the rate of purine alkaloid synthesis from AICA-riboside. It was therefore concluded that IMP newly synthesized by the *de novo* pathway is utilized directly for theobromine biosynthesis in young tea leaves. These results do not rule

out the participation of caffeine biosynthetic pathways from preformed purines, but the data suggest that the *de novo* pathway is involved in the caffeine biosynthesis in young growing tea leaves, in which a very rapid net accumulation of purine alkaloids is observed.

6.2. *S*-Adenosyl-L-methionine (SAM) cycle

Koshiishi *et al.* (58) propose a pathway in which adenosine, released from SAH, is converted to xanthosine via adenine, AMP, IMP and XMP. The formation of caffeine by this pathway is closely associated with the "SAM cycle" (also known as the "activated-methyl cycle") because the three methylation steps in the caffeine biosynthetic pathway all use SAM as the methyl donor (Figure 4). In the process, SAM is converted to SAH, which is in turn hydrolyzed to L-homocysteine (Hcy) and adenosine. The adenosine is used to synthesize the purine ring of caffeine, while Hcy is recycled to replenish SAM levels. Since three moles of SAH are produced via the SAM cycle for each mole of caffeine that is synthesized, this pathway has the capacity to be the sole source of both the purine skeleton and the methyl groups required for caffeine biosynthesis in young tea leaves.

6.3. Degradation of adenine nucleotides

Structurally, xanthosine can be produced in adenine nucleotide catabolism, although the major catabolic pathway of AMP does not include this metabolite. Therefore, part of the xanthosine used for caffeine biosynthesis may be derived from the adenine nucleotide

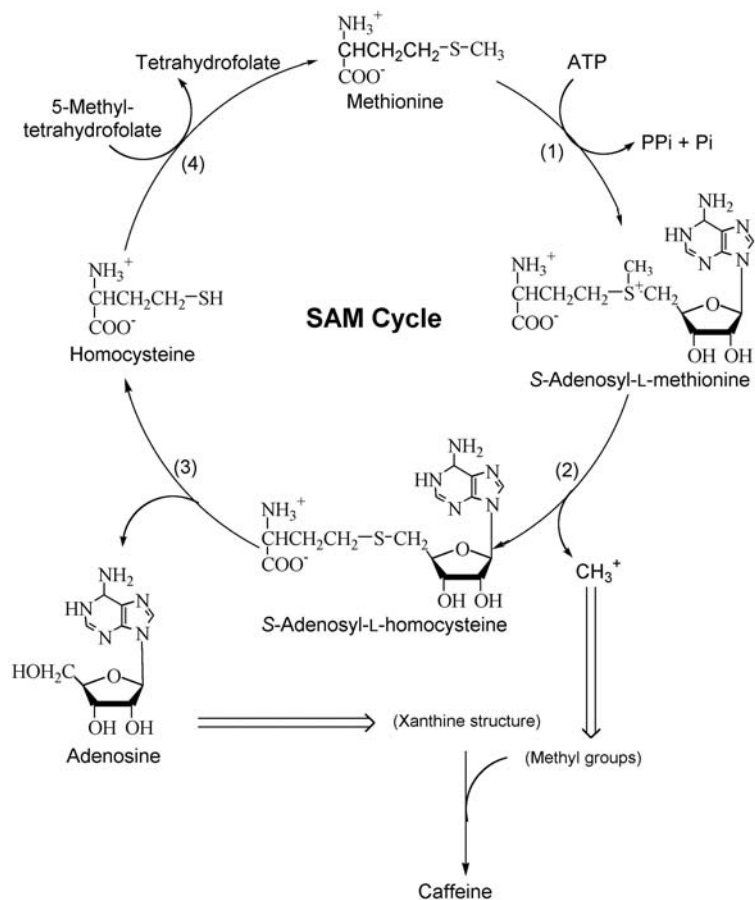


Figure 4. The SAM cycle (the activated methyl cycle) in plants. Enzymes: (1) SAM synthetase (EC 2.5.1.6), (2) SAM-dependent *N*-methyltransferases, (3) S-adenosylhomocysteine (SAH) hydrolase (EC 3.3.1.1), (4) Methionine synthase (EC 2.1.1.13). Adenosine released from the cycle is salvaged to adenine nucleotides and utilised both for purine structure of caffeine via xanthosine and for re-synthesis of SAM via ATP.

pool. There are five possible pathways for xanthosine synthesis from AMP, but the AMP → IMP → XMP → xanthosine pathway is the most likely route (Steps 4-6, in Figure 2). All three enzymes, AMP deaminase, IMP dehydrogenase and 5'-nucleotidase, have been detected in tea leaves (58). The pool of adenine nucleotides is larger than that of guanine nucleotides in all purine alkaloid-forming plants examined (44, 63), so that the contribution of adenine nucleotides to caffeine biosynthesis may be larger than that of guanine nucleotides.

6.4. Degradation of guanine nucleotides

Several reports demonstrate the biosynthesis of caffeine from [8-¹⁴C] guanine and [8-¹⁴C]guanosine (5, 45). The data indicate that caffeine is also produced from guanine nucleotides. Conversion of guanine to GMP is catalyzed by hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8). Xanthosine, which is the initial methyl acceptor of the caffeine biosynthesis, seems to be formed from GMP by a GMP → guanosine → xanthosine pathway (Steps 7-8 in Figure 2). Negishi *et al.* (64) found guanosine deaminase in cell-free extracts from young tea leaves.

7. DIVERSITY OF PURINE ALKALOID BIOSYNTHESIS

7.1. Differences in end-products

Three different groups of purine alkaloid-accumulating plants can be distinguished according to the accumulated purine alkaloids. These are caffeine plants (tea, coffee, and maté), theobromine plants (cacao, cocoa tea and *Camellia irrawadiensis*) and methyluric acid plants (*Coffea dewevrei* and kucha).

7.2. Purine alkaloid synthesis in theobromine plants

In very young cacao leaves, ¹⁴C-labelled adenine, adenosine, guanine, guanosine, hypoxanthine and inosine are converted to salvage products (nucleotides and nucleic acids), degradation products (ureides and CO₂) and purine alkaloids (3- and 7-methylxanthine, 7-methylxanthosine and theobromine). In contrast, ¹⁴C-labelled xanthine and xanthosine were not salvaged, and nearly 20% of [8-¹⁴C]xanthosine was converted to purine alkaloids (35). These observations are consistent with the following biosynthetic pathways for theobromine: (a) AMP → IMP → XMP →

xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine; (b) GMP → guanosine → xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine. These pathways are the same as those observed in caffeine plants, such as tea and coffee. Although no caffeine from ¹⁴C-labelled purine bases and nucleosides was observed during 18 h-incubations, exogenously supplied [8-¹⁴C]theobromine was converted to caffeine in young leaves. Conversion of theobromine to caffeine may therefore be slow in cacao leaves. The ability to synthesize purine alkaloids disappeared in the subsequent growth stages of cacao leaves. Tracer experiments with [8-¹⁴C] adenine using cacao fruits at various growth stages indicate that a major site of theobromine synthesis is the young pericarp and cotyledons of growing fruits. Limited amounts of purine alkaloids may be transported from the pericarp to seed tissue, but most purine alkaloids accumulating in seeds appears to be synthesized in cotyledons (34). Extraction of *N*-methyltransferases from cacao leaves and fruits has been attempted, but no activity was found in the resulting cell-free preparations (unpublished observation).

Camellia irrawadiensis leaves contain theobromine and little or no caffeine, and young leaves convert both [8-¹⁴C]adenine and [8-¹⁴C]hypoxanthine to theobromine, without detectable incorporation of radioactive labels into caffeine (31). A similar conversion of [8-¹⁴C] adenine occurs in stamens and petals isolated from *Camellia irrawadiensis* flower buds, in which ca. 40% of the radioactivity taken up by these organs is recovered as theobromine (30). The obvious explanation for the lack of caffeine in *Camellia irrawadiensis* is limited theobromine *N*-methyltransferase activity. This is yet to be verified since presumably for technical reasons, enzyme preparations from young leaves of *Camellia irrawadiensis* show no detectable *N*-methyltransferase activity.

Caffeine is also not detectable in leaves of cocoa tea (*Camellia ptilophylla*) which contain theobromine, of which the highest concentrations, together with trace amounts of theophylline, are found in young expanding leaves (65). Incubation of young *Camellia ptilophylla* leaves with [8-¹⁴C] adenine caused in 10% of the absorbed radioactivity to be recovered as theobromine. The substrate specificity of *Camellia ptilophylla* *N*-methyltransferase activity was examined using gel-filtrated extracts from young leaves. Activity was detected with 7-methylxanthine as a methyl acceptor, but not with dimethylxanthines, i.e., theobromine, theophylline, and paraxanthine. The major caffeine biosynthesis pathway via theobromine is therefore blocked; this was confirmed by the observation that leaf segments of *Camellia ptilophylla* could not convert [2-¹⁴C]theobromine to caffeine. Substrate specificity of the *Camellia ptilophylla* *N*-methyltransferase activity is such that, as well as blockage of the main biosynthesis pathway, the alternative minor routes to caffeine, via substrates such as paraxanthine, are also likely to be blocked. Cocoa tea leaves possess enzymes which convert caffeine to theobromine, so that even if a small amount of

caffeine were synthesized, it would be immediately converted to theobromine (65).

7.3. Methyluric acid synthesis

Theacrine was discovered as a minor component of *Camellia sinensis* leaves by Johnson (66). It has since been detected along with related methyluric acids, such as liberine (*O*(2), 1, 9-trimethyluric acid) and methyl liberine (*O*(2), 1, 7, 9-tetramethyluric acid) in several species of coffee including *Coffea liberica*, *Coffea dewevrei* and *Coffea abeokuta* (24, 25, 67), as well as in seeds of various *Herrania* and *Theobroma* species (36, 37). Petermann and Baumann (25) showed that caffeine is converted to liberine via theacrine and methyl liberine in leaves of these three *Coffea* species.

Zheng *et al.* (29) recently investigated theacrine synthesis in leaves of an unusual Chinese tea, *Camellia assamica* var. *kucha*. They showed that theacrine is synthesized from adenosine via caffeine. Radioactivity from [methyl-¹⁴C] SAM was incorporated into theacrine as well as into theobromine and caffeine by leaf disks of *kucha*, indicating that SAM acts as the methyl donor not only for caffeine biosynthesis but also for theacrine production. [8-¹⁴C]Caffeine was converted to theacrine by *kucha* leaves, with greatest incorporation in expanding buds. When [8-¹⁴C] adenosine was incubated with young *kucha* leaves for 24 h, up to 1% of the total radioactivity was recovered in theacrine. However, pulse-chase experiments with [8-¹⁴C] adenosine demonstrated much more extensive incorporation of label into caffeine than into theacrine, possibly because of dilution of [¹⁴C]caffeine by the large endogenous caffeine pool. These results indicate that theacrine is synthesized in *kucha* leaves from caffeine in what is probably a three-step pathway, with 1, 3, 7-methyluric acid as an intermediate.

8. SUBCELLULAR DISTRIBUTION AND TRANSPORT OF PURINE ALKALOIDS

Subcellular compartmentation of purine alkaloids has not yet been verified. However, it is in keeping with many other secondary metabolites that caffeine and other purine alkaloids are stored in vacuoles (68). The mechanisms for uptake and sequestration of caffeine in vacuoles is yet to be investigated. Active/passive transport by channels and/or transporters and membrane/vacuole fusion, may be involved in these processes. Binding of caffeine to polyphenols such as catechins (tea) and chlorogenic acids (coffee) may allow the accumulation of these compounds in vacuoles against a concentration gradient. Baumann and co-workers suggested that vacuolar compartmentation of purine alkaloids depends exclusively on the formation of complexes with chlorogenic acids (69, 70).

Compartmentation of enzymes of caffeine biosynthesis and the SAM cycle has been examined biochemically (58, 71). It was found that caffeine synthase, which is a key enzyme catalysing the final two steps of caffeine biosynthesis, is located in chloroplasts.

In addition to the caffeine synthase activity, more than 70% of the total activity of SAH hydrolase and parts of adenine salvage enzyme activities (adenosine kinase, adenosine nucleosidase and adenine phosphoribosyltransferase) were associated with Percoll-purified chloroplasts. In contrast, SAM synthetase was detected exclusively in the cytosol. Recently, genes encoding SAH hydrolase have been cloned from tobacco and parsley. The *N*-terminal sequences lack the typical features of transit peptide signals, suggesting that the genes encode the cytosol form of SAH hydrolase in these plants (see references in 58). However, the data presented by Koshiishi *et al.* (58) show that tea SAH is more likely to be a chloroplastic enzyme. Since SAM synthetase is a cytosolic enzyme, SAM must be produced in the cytosol of tea leaves. Nothing is known about the subcellular localisation of methionine synthetase. Koshiishi *et al.* (58) proposed a model in which homocysteine produced in chloroplasts is transported to the cytosol, but transport of methionine cannot be excluded. Conversion of methionine to SAM takes place in the cytosol of young tea leaves, and SAM returns to chloroplasts where it serves as a methyl donor in caffeine biosynthesis; in the process it is converted to SAH. The available evidence also suggests that adenosine derived from SAH is converted to adenine and AMP in tea chloroplasts.

Examination of the subcellular localization of theobromine synthase (CaMXMT) using the fusion protein of CaMXMT and GFP by contrast found it present predominantly in the cytoplasm of onion epidermal cells (11). The PSORT program running with the deduced amino acid sequence also predicted a high possibility of cytosolic localization for CaMXMT. Based on these results, Ogawa *et al.* (11) suggested that caffeine biosynthesis occurs in the cytoplasm (cytosol) of cells in buds and young leaves.

The observed discrepancy in the localization of the caffeine biosynthetic pathway according to the different techniques is not yet resolved.

9. POSSIBLE FUNCTION OF PURINE ALKALOIDS IN PLANTS

The physiological role of endogenous purine alkaloids and related compounds in higher plants remains undetermined. Degradation of caffeine is relatively slow even in aged leaves of most species, and it appears not to act as a nitrogen reserve since considerable amounts remain in leaves after abscission. Two hypotheses have been prepared concerning the role of caffeine in plants. The "chemical defense theory" proposes that the high concentrations of caffeine in young leaves, fruits and flower buds of species such as *Coffea arabica* and *Camellia sinensis* act as a chemical defense to protect young soft tissues from predators, such as insect larvae (72-75). The "allelopathic or autotoxic function theory" proposes that caffeine in seed coats is released into the soil to inhibit germination of other seeds (76, 77). Though these hypotheses are

interesting, there is little evidence that they actually operate in nature.

10. BIOTECHNOLOGY OF PURINE ALKALOIDS

10.1. Caffeine production in cell and tissue cultures

Caffeine is in demand for medicines; aspirin tablets containing caffeine are being sold on an increasing basis, and large amounts of caffeine are used to supplement soft drinks. The caffeine required for these products is obtained either by large scale chemical synthesis, which is relatively straightforward, or as a byproduct of procedures used to decaffeinate tea and coffee (78). Caffeine production by tissue and cell cultures of coffee and tea has had little economic impact, although such systems could prove very useful in studies of the mechanisms involved in purine alkaloid biosynthesis.

10.2. Decaffeinated beverages

In economic terms, coffee is one of the most valuable agricultural products exported by the third world and developing countries in Central and Southern America and Africa. *Coffea arabica* (Arabica coffee) is cultivated extensively and represents ca. 70% of the market. The remaining 30% consists mainly of *Coffea canephora* (Robusta coffee). Beans of Arabica and Robusta coffee respectively contain ca. 1% and 2% caffeine. Since the early 1970s, demand for decaffeinated coffee has increased rapidly, currently accounting for more than 10-20% of coffee sales. This is because of a growing belief that ingestion of large amounts of caffeine, which is a stimulant, has adverse effects on health. This has led to extensive debate in the medical literature with no clear conclusions being drawn. In the long term, it seems that the increasing demand for decaffeinated coffee would be better met by the use of *Coffea* species with beans that contain significantly lower levels of caffeine than *Coffea arabica* or *Coffea canephora*.

Since *Coffea arabica* is polyploid and most other species of *Coffea* are diploid ($n = 22$), there are also genetic barriers that prevent effective crossings between *Coffea arabica* and other *Coffea* species, such *Coffea salvatrix* and *Coffea bengelensis*, that contain much lower levels of caffeine. The use of genetic engineering to produce transgenic caffeine-deficient *Coffea arabica* may ultimately prove to be a more practical than breeding programmes.

As stated earlier, genes encoding *N*-methyltransferases have been cloned. This development makes genetic engineering possible to produce transgenic tea and coffee plants that are naturally deficient in caffeine. The use of such products to make full flavored caffeine-free beverages will be of interest to the increasing number of consumers who are concerned about the potentially adverse effects of caffeine consumption on their health. Genetic engineering to produce transgenic caffeine-deficient tea and coffee might ultimately prove to be a more practical proposition.

The cloning of genes related to the caffeine biosynthesis (*N*-methyltransferase genes) is an important advance towards the production of transgenic caffeine-deficient tea and coffee through gene silencing with antisense mRNA or RNA interference technology. A possible complication is that transgenic plants might accumulate xanthosine instead of caffeine. Instead of accumulating, xanthosine might be converted to xanthine, which would be degraded by the purine catabolism pathway. Recently, genes encoding *N*-methyltransferases for caffeine biosynthesis have been cloned (7-12), and transgenic caffeine-deficient *Coffea canephora* plants created (79).

Keya *et al.* (80) suggested that suppression of IMPDH gene expression is an alternative way to produce decaffeinated transgenic tea and coffee plants. Koshiishi *et al.* (63) have shown that nucleotide profiles of tea leaves are similar to profiles of other plant species. It is currently believed that the tea-specific amino acid, theanine, makes a major contribution to umami taste. IMP may also be involved, since it has been shown that nucleotide seasonings interact synergistically with amino acid-based tastes. Purine nucleotides in fresh tea leaves may be converted to IMP during commercial processing (63). In that case, metabolic engineering to accumulate IMP and related nucleotides would be of value. Kaya *et al.* (80) indicate that caffeine synthesis should be reduced and free purine nucleotides, including IMP, will accumulate if IMPDH activity is blocked. Transgenic tea plants with reduced IMPDH activity therefore offer the intriguing prospect of a beverage with a low caffeine content coupled with enhanced flavour quality.

11. SUMMARY AND PERSPECTIVE

The major route to caffeine in higher plants is a xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway. Several minor biosynthesis and pathways also operate, probably because of the broad substrate specificity of *N*-methyltransferases. Diversity of caffeine metabolism between different purine alkaloid-containing plants appears to be the result of variations in the substrate specificity of the *N*-methyltransferases in different species. Caffeine is synthesized in young tissues, and caffeine synthase resides in chloroplasts in developing leaves. The precursors of caffeine are derived from purine nucleotides which originate from *de novo* purine biosynthesis, as well as adenine and guanine nucleotides pools which are formed by the salvage pathways of adenosine, adenine and other related compounds. Adenosine released from the SAM cycle may be salvaged to AMP and utilized for the synthesis of the purine ring in caffeine biosynthesis, although some adenosine is salvaged to ATP and is utilized for SAM synthesis from methionine. The rate of caffeine biosynthesis appears to be regulated primarily by the induction and repression (so-called "coarse control") of *N*-methyltransferases, especially 7-methylxanthosine synthase and caffeine synthase, and by the activation and inhibition of these *N*-methyltransferase activities by the SAM/SAH ratio and the availability of purine precursors ("fine control"). Some reports now exist of the

molecular biology and genetic engineering of caffeine biosynthesis (See later chapter written by Kato and Mizuno). Studies of caffeine are therefore entering a new phase in which the major challenge will be to understand the molecular mechanisms that regulate purine alkaloid metabolism. These developments promise the biotechnology to produce caffeine-deficient transgenic *Coffea arabica* and *Camellia sinensis* plants that will meet the growing demand for decaffeinated coffee and tea.

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