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N-Methyltransferase Activities in Suspension Cultures of *Coffea arabica* L.

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ABSTRACT

Suspension cultures of Coffea arabica L. are a useful source for methyltransferase preparations of high activity catalysing the transfer of methylgroups from S-adenosyl-L-methionine to 7-methylxanthine and to theobromine producing theobromine and caffeine respectively. Surprisingly, these enzyme activities are not correlated with the availability of precursors during a culture cycle. They are highest in the growth phase when supply of precursors is reduced. Mixed substrate experiments and time dependent changes in the enzyme activity ratio provide indirect evidence for the existence of two separate enzymes catalysing the final methylations in caffeine biosynthesis.

INTRODUCTION

Alkaloid concentration (% of DW) achieved by in vitro cultivation of plant tissues is generally distinctly lower than in the original plant. Among the few exceptions are the purine alkaloids theobromine and caffeine (Fig.1), which are produced by callus and suspension cultures of Coffea arabica L. and C. canephora Pierre ex Froehner in concentrations almost as high as in the plant (Keller et al. 1972, Frischknecht et al. 1977, Frischknecht and Baumann 1980, Baumann and Frischknecht 1982). One of the explanations for this ready formation are the possibly close but only partially known connections of these secondary plant substances to adenine-containing compounds of primary metabolism (Suzuki and Takahashi 1976, Baumann et. al 1978). More substantial is the observation that in suspension cultures established from a variety of purine alkaloid-producing plants, high productivity goes along with a low caffeine degradation activity and vice versa (Baumann and Frischknecht 1982). Moreover, high and low producing culture strains of Coffea arabica differ markedly in their biotransformation capacity of theobromine to caffeine, indicating different levels of methyltransferase activities (Frischknecht and Baumann 1980).

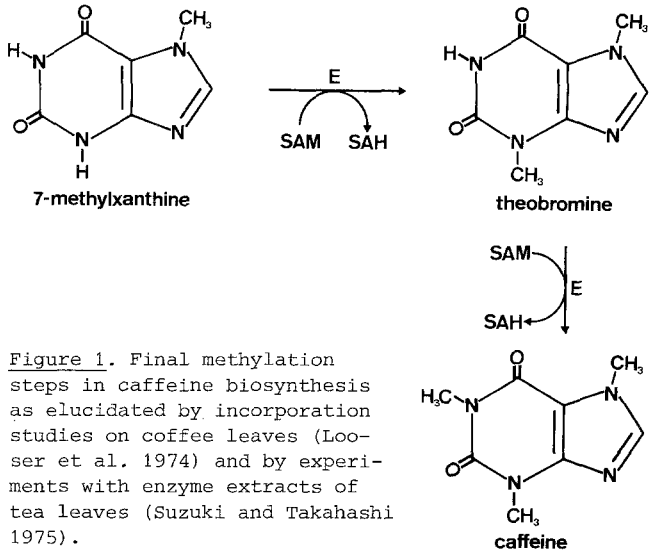

Figure 1. Final methylation steps in caffeine biosynthesis as elucidated by incorporation studies on coffee leaves (Looser et al. 1974) and by experiments with enzyme extracts of tea leaves (Suzuki and Takahashi 1975).

This publication describes the methyltransferase activities towards 7-methylxanthine and theobromine (Fig.1) in relation to culture growth and to purine alkaloid production. The physiological and metabolic conditions which would provide optimal alkaloid productivity are discussed.

MATERIALS AND METHODS

Cell Cultures

Suspension cultures of Coffea arabica L. were established from internodes via callus cultures as already described (Frischknecht et al. 1977, Frischknecht and Baumann 1980). There is a positive correlation between alkaloid production and aggregate size. To couple reasonable productivity with reproducibility we used cultures of medium-sized (ca 5 mm) aggregates. For time-course experiments such established suspension cultures were combined and then portioned into six parallel cultures, each containing initially 3 g of cell material suspended in 16 ml medium.

Enzyme preparation

Cell cultures (3 to 8 g) were homogenized in a mortar in the presence of liquid N_2. To the resulting powder twice its weight of a 0.1M K-phosphate buffer solution (pH 7.3, mercaptoethanol 5mM, Na_2EDTA 5 mM, vitamin C 0.5 %) was added. After addition of 0.3-0.5g polyvinylpolypyrrolidone (Sigma Chemical Co.,St.Louis, USA), previously hydrated with buffer, the slurry was thoroughly mixed and centrifuged at 20,000 g for 20 min at 4°C. An aliquot of the supernatant (crude extract) was then treated with a saturated ammonium sulfate solution at 0°C. The precipitate obtained at 50 % saturation was recovered by centrifugation, redissolved in the extracting buffer (half the original volume of the supernatant aliquot) and immediately used for the enzyme assays.

Standard Enzyme Assay

The reaction mixture (500 µl) included 250 µl of the ammonium sulfate precipitated cell-free extract containing about 1 mg protein per ml, 50 µl 2 mM $MgCl_2$, 18.5 kBq S-adenosyl-L-[methyl-^{14}C]methionine (1.88 GBq per mmol) in 50 µl 0.5 M K-phosphate buffer pH 7.3, 100 µl 0.5 M K-phosphate buffer and 50 µl of the appropriate methylxanthine (in H_2O). The final concentrations (mM) were: K-phosphate 200, vitamin C 14, mercaptoethanol 2.5, Na_2EDTA 2.5, $MgCl_2$ 0.2 and methylxanthine 0.2. After incubation for 60 min at 30°C the reaction was stopped by cooling and by the addition of 0.5 ml $CHCl_3$. First the aqueous and then the chloroform phase were applied to a diatomaceous earth column (Extrelut, Merck, Darmstadt, FRG; 0.3 g). The methylated xanthines were eluted with 4.5 ml $CHCl_3$. Following solvent evaporation the residue was dissolved in $CHCl_3$ and chromatographed on TLC plates (silica gel F254, Merck) with $CHCl_3$/MeOH (9:1) as solvent. Radioactivity was located by radiochromatogram scanning, eluted with 2 ml MeOH and measured by liquid scintillation counting. Bq values were calculated by the channel ratio.

Kinetic Constants

A modified standard assay (500 µl, pH 7.3) was used consisting of S-adenosyl-L-[methyl-^{14}C]methionine (1.88 GBq/mmol, final concentrations 0.043, 0.083, 0.18 and 0.39 mM), of the corresponding methylxanthine (final concentrations 0.1, 0.2, 0.4 and 0.8 mM) and of the enzyme extract (100 µl). The concentrations (mM) of the other components were: K-phosphate 170, vitamin C 5.6, mercaptoethanol 1.0, Na_2EDTA 1.0 and $MgCl_2$ 0.2. The reaction time was 5 min.

Purine Alkaloids

They are dispersed equal to the ratio of volume tissue to volume nutrient medium indicating a free exchange in this system. For determination samples of culture medium were chromatographed on a data processing HPLC-system (Waters Associates) equipped with a 4 x 300 mm column (10 um LiChrosorb RP-18, Merck) and with a UV detector monitoring the eluant at 271 nm. Theobromine (k'=0.8) and caffeine (k'=3.7) were eluted with 25 % MeOH. When quantification of 7-methylxanthine was required, an 18 % MeOH eluant, buffered at pH 5.7 (20 mM K-phosphate), was used. The k' values were 1.0, 2.6 and 11.5 for 7-methylxanthine, theobromine and caffeine respectively.

RESULTS AND DISCUSSION

Stability, pH profile, effects of inhibitors and of substrate concentration (apparent K_m values) were determined for the methyltransferase activity of Coffea arabica (Roberts and Waller 1979). Cell-free extracts with significant activity were obtained only from the young green coffee fruits and with the use of polyvinylpolypyrrolidone in an atmosphere of N_2. Moreover, the authors noticed a rapid loss in enzyme activity which could be reduced by the addition of bovine serum albumine. In contrast to this, extracts from suspension cultures, even though they were prepared without polyvinylpolypyrrolidone, contain high and relatively stable methyltransferase activity. The rates of formation were found to be constant as regards time up to at least 90 min under the conditions of standard enzyme assay described in Materials and Methods. The K_m values determined by the method of Florini and Vestling (1957) for 7-methylxanthine and S-adenosyl-L-methionine were 0.1 and 0.005 mM respectively, and for theobromine and S-adenosyl-L-methionine 0.2 and 0.016 mM respectively. For both methylations an intersecting initial velocity pattern was obtained leading us to assume a sequential mechanism in which a ternary substrate-enzyme complex is formed before either product is released (Cleland 1970).

As was already noticed earlier by Frischknecht and Baumann (1980), a considerable amount of alkaloid is produced after termination of the growth phase (Fig. 2A), contrary to expectations as to the time course of the methyltransferase activities (Fig. 2B). Highest caffeine production is found between d 7 and 9 when the enzyme

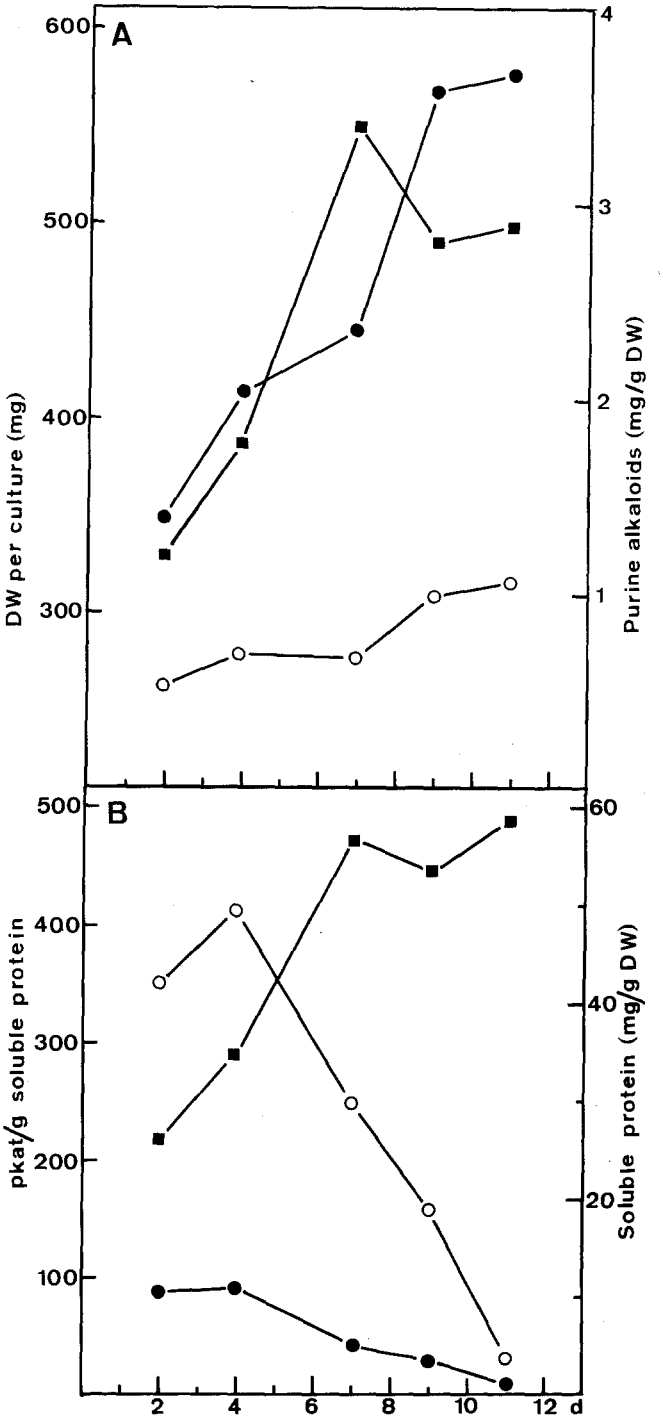

Figure 2. A: Relative theobromine (O —— O) and caffeine (● —— ●) content during growth (■ —— ■) of Coffea arabica suspension cultures. The relative content of 7-methylxanthine determined in a separate experiment dropped after 3 d from an initial value of 0.11 mg to a constant level of 0.05 mg. B: Time course of soluble protein (■ —— ■) (Schaffner and Weissmann 1973) and of methyltransferase activities (standard assay) towards 7-methylxanthine (O —— O) and theobromine (● —— ●).

activity for methylation of theobromine to caffeine decreases considerably. The discrepancy is even more pronounced in case of relative theobromine formation, which is zero at a time of very high enzyme activity towards 7-methylxanthine (d 4 to 7). Obviously there is a shortage of precursors (methylxanthines and/or methyldonors) during growth. This interpretation is strengthened by the fact that the velocity of caffeine formation is increased by a factor of 3 and 1.5, when theobromine is added during growth and in the stationary phase respectively(Frischknecht and Baumann 1980). In this way, in tissue culture a maximum production rate of more than 1,000 µg caffeine per d and g DW was reached, a value characteristic for very young expanding leaves of plants growing under favourable physiological conditions (Frischknecht et al. 1982). We have repeatedly observed that in such leaves the rate is not enhanced in any degree by exogeneous theobromine, most likely because of the existing large pool.

For the in vitro situation it can be concluded that cells in the growth phase exhibit high methyltransferase activities but produce a short supply of purine ring for secondary metabolism.Later on,when the primary processes are reduced, a surplus of purine metabolites meets with comparably low methyltransferase activities. Since there is a free exchange of caffeine and of its immediate precursors between tissue and medium (Frischknecht and Baumann 1980), productivity might be improved by a system in which cultures of different growth stage ("exponential" and stationary) are linked with a circulating medium. However, the requirement for the second substrate, S-adenosyl-L-methionine, has to be determined likewise.

During cultivation the methyltransferase activity ratio (7-methylxanthine to theobromine) increases from 4.0 to a maximum value of 6.0 at d 7 and finally drops to 3.8. This growth stage dependent change in the activity ratio points to the existence of two discrete methyltransferases. In Table 1 the methyltransferase activities towards equimolar mixtures of two methyl accepting substrates are compared with those obtained with each substrate separately, at the same individual concentration. In a one-enzyme system individual velocity would be reduced in the presence of two substrates on account of competition for the active site. For the 7-methylxanthine/theobromine test no mutual effect on the methylation rates is observed: The sum of the activities measured in a mixture of both is not significantly different from the activity determined for 7-methylxanthine alone. In the 7-methylxanthine/paraxanthine test the formation of caffeine is not altered by the presence of 7-methylxanthine. Paraxanthine, which is a highly reactive methylacceptor in the methyltransferase reaction but has proved not to be a natural substrate in caffeine biogenesis (Suzuki and Takahashi 1975), acts as a strong inhibitor of the reaction 7-methylxanthine → theobromine. On the other hand, the methylation of theobromine to caffeine is not significantly inhibited by paraxanthine. It must therefore be assumed that two separate enzymes are involved in the final steps of caffeine biosynthesis, one mediating the transfer of a methyl group from S-adenosyl-L-methionine to 7-methylxanthine and the other to theobromine.

Table 1. Methyltransferase activities determined towards two different substrates and an equimolar mixture of these. For each substrate combination one enzyme extract from a separate culture was prepared. Except substrate concentration (0.2 mM) standard assay conditions were used. Paraxanthine = 1,7-dimethylxanthine.

Substrate	Product radioactivity (Bq per assay)	
	Theobromine	Caffeine
7-Methylxanthine	528	
Theobromine		177
7-Methylxanthine + theobromine	474	156
7-Methylxanthine	529	
Paraxanthine		1,935
7-Methylxanthine + paraxanthine	204	2,022
Theobromine		133
Paraxanthine		1,080
Theobromine + paraxanthine		1,004

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