Pergamon 0031-9422(95)00185-9 Phytochemistry, Vol. 39, No. 6, pp. 1363–1370, 1995

0031-9422/95 $9.50 + 0.00

ARE XANTHOSINE AND 7-METHYLXANTHOSINE CAFFEINE PRECURSORS?

BRIGITTE H. SCHULTHESS and THOMAS W. BAUMANN*

Institute of Plant Biology, University of Zurich, CH-8008 Zurich, Switzerland

(*Received in revised form* 7 *February* 1995)

Key Word Index—*Coffea arabica*; Rubiaceae; coffee; tissue culture; incorporation studies; caffeine biosynthesis; purine metabolism; adenine; xanthosine; 7-methylxanthosine.

Abstract—Although caffeine biosynthesis has been reported to start with the methylation of xanthosine to yield 7-methylxanthosine, we failed to demonstrate in suspension-cultured cells of coffee the presence of 7-methylxanthosine, even after considerable stimulation of overall purine alkaloid (PA) formation by means of ethephon or/and adenine. Hence, in order to increase sensitivity and to study incorporation kinetics, stimulation was combined in the present work with radioactive labelling; [^{14}C]adenine or [methyl-^{14}C]methionine was fed to cells with PA synthesis enhanced either by 5 mM ethephon or by 1 mM adenine. Ethephon treatment resulted in a high (50 to 80% after 24 hr) incorporation of adenine radioactivity into PA, whereas pre-incubation with 'cold' adenine known to increase long-term PA formation strongly reduced that incorporation in favour of labelling xanthosine and related nucleosides. Neither ethephon nor adenine treatment led to a distinct incorporation of [^{14}C]adenine or [methyl-^{14}C]methionine into 7-methylxanthosine. Traces of [^{14}C]7-methylxanthosine (less than 1%) could only be detected in cells pre-incubated with 1 mM adenine and thus exhibiting 'artificially' enlarged endogenous xanthosine pools either strongly labelled (after [^{14}C]adenine-feeding) or unlabelled (after [methyl-^{14}C]methionine-feeding). Kinetic analyses upon labelling with [^{14}C]adenine revealed that, based on the specific radioactivity, xanthosine must be excluded as a precursor in caffeine biosynthesis, unless we consider separate pools of xanthosine, one being a collecting pool, the other the PA biosynthesis pool. Therefore, if we adhere to the xanthosine hypothesis, we should postulate metabolic channelling of the early events in PA biosynthesis, including formation of xanthosine, its methylation to 7-methylxanthosine, and finally enzymatic hydrolysis to 7-methylxanthine. However, currently there is no argument against the possibility of the first methylation taking place at the nucleotide level.

INTRODUCTION

In tea (*Camellia sinensis*), as well as in coffee, caffeine has been reported to be synthesized from xanthosine *via* 7-methylxanthosine, 7-methylxanthine and theobromine, with S-adenosylmethionine (SAM) as the source of all three methyl groups [reviewed in 1]. Negishi and coworkers were the first to demonstrate *in situ* formation of labelled 7-methylxanthosine, 7-methylxanthine, theobromine and caffeine from [^{14}C]xanthosine in tea [2] and coffee shoots [3].

In a first approach, we tried without success to demonstrate the *in situ* presence of 7-methylxanthosine by means of HPLC/UV and using stimulators of caffeine biosynthesis [4]. Caffeine formation in suspension-cultured cells was strongly enhanced by ethephon (2-chloroethylphosphonic acid, an ethylene-releasing compound) and adenine. Thereby, 7-methylxanthine was accumulated by a factor of 14 (10 mM ethephon) and the xanthosine level was temporarily increased by a factor of 45 (1 mM adenine). Although the experimentally induced metabolic situation clearly should favour the synthesis of 7-methylxanthosine, this compound was not to be found. Furthermore, the existence of 7-methylxanthosine could not be verified by other research groups even after feeding ring-labelled [^{14}C]adenine or [methyl-^{14}C]methionine, two well-known efficient precursors of caffeine biosynthesis [5–13].

To resolve this inconsistency, we combined, in the present study, radioactive labelling with exogeneous stimulation of caffeine biosynthesis in suspension-cultured cells of coffee. Labelled precursors were [^{14}C]adenine or L-[methyl-^{14}C]methionine, which, after uptake, first have to be 'activated' by primary metabolism to AMP and SAM, respectively. Hence, their incorporation into caffeine is merely the result of an unspecific biotransformation as may be argued for xanthosine feeding. Special attention was paid to the occurrence of labelled 7-methylxanthosine, as well as to the specific activity of the putative precursor xanthosine and of the purine alkaloids (PA), 7-methylxanthine, theobromine and caffeine. Three different experimental sets were used in combination with [^{14}C]adenine feeding:

*Author to whom correspondence should be addressed.

(1) PA formation was experimentally enhanced by 5 mM ethephon;

(2) cellular pools of purines were expanded by pre-incubation with 1 mM adenine allowing an accurate determination of their concentrations and thus of specific activities;

(3) a coffee cell-line devoid of any PA production was compared with the 'normal' PA-producing line.

Similar experiments were also carried out with L-[methyl-^{14}C]methionine, in order to intensify the selective search for 7-methylxanthosine or any unknown methylated purine.

RESULTS AND DISCUSSION

Studies with [^{14}C]adenine

To initially study and optimize uptake of [^{14}C]adenine, we fed cells, pre-cultured for 5 days, with 4 μCi of either [U-^{14}C]adenine (270 mCi mmol^{-1}, 3.7 μM) or [8-^{14}C]adenine (49 mCi mmol^{-1}, 20.4 μM), respectively. Within 30 min, [U-^{14}C]adenine was completely taken up but [8-^{14}C]adenine only partially (38%). Thus, due to the lower concentration required, feeding [U-^{14}C]adenine turned out to be superior, especially in the case of pulse-chase experiments, where a much higher dilution with 'cold' adenine can be achieved.

Pulse-chase studies are considered most suitable for elucidating a biosynthetic pathway due to improved resolution of sequential product labelling by the chase. However, the chase with 0.4 mM 'cold' adenine 1 hr after feeding [U-^{14}C]adenine resulted after 24 hr, as compared to the pulse alone, in a ($ca \times 2.5$) lower incorporation into PA and in an increase of radioactivity into purine nucleosides; in xanthosine it was by a factor of 7. Since [^{14}C]adenine was rapidly metabolized (only *ca* 6% of the applied radioactivity was found as adenine after 30 min, i.e. when [^{14}C]adenine uptake was completed), this unexpected result can only be explained by expansion of the adenine metabolite pools effected by the 'cold' adenine [4]. Obviously, as will be discussed in detail below, the label from [^{14}C]adenine was captured in these pools and consequently incorporation into PA was lowered.

Based on these initial optimizing studies (see also Experimental) and in order to achieve maximum incorporation rates into PA, cell cultures were subsequently only pulse-fed with [U-^{14}C]adenine after a 5-day preculture.

Table 1. Percent incorporation of [U-^{14}C]adenine into purine metabolites by cell-line E and M of *Coffea arabica* after 24 hr

Cell-line Pre-incubation	E Control (%)	M Control (%)	M 5 M Ethephon (%)	M 1 mM Adenine (%)
7-Methylxanthine	0	1.23	11.49	0.38
Theobromine	0	3.38	21.12	1.33
Caffeine	0	26.62	16.55	9.34
Σ Purine alkaloids	**0**	**31.23**	**49.16**	**11.05**
Adenosine	2.31	10.25	0.46	5.76
Guanosine	1.00	1.25	0	1.27
Xanthosine	11.90	8.28	12.79	45.69
Σ Nucleosides	**15.21**	**19.78**	**13.25**	**52.72**
AMP	14.65	4.69	5.12	8.10
GMP	2.55	0.55	0.58	0
Σ Nucleotides	**17.20**	**5.24**	**5.70**	**8.10**
Allantoin	39.84	28.50	15.64	11.56
Uric acid	14.79	4.23	7.88	3.84
Σ Purine catabolites	**54.63**	**32.73**	**23.52**	**15.40**
SAM	1.38	1.94	1.06	1.18
NAD	5.91	2.71	3.29	3.21
7-Glucopyranosyl adenine	0.87	3.26	1.87	4.88
Adenine	0	1.04	0	1.38
Total radioactivity	**95.20**	**97.93**	**97.85**	**98.02**

Cells (3 g) of a caffeine-producing (M) and a caffeine-free (E) coffee cell-line, pre-incubated during 5 days with 1 mM adenine, 5 mM ethephon or no supplement (control) were fed with 4 μCi [U-^{14}C]adenine suspended in 1 ml medium. After 24 hr, cells were harvested, extracted and analysed by HPLC with on-line radiodetection. Inosine, ATP and MTA were only labelled shortly after feeding and are therefore not listed.

Cells used for feeding experiments were pre-incubated during 5 days at a ratio of 1:1 with medium containing either 1 mM adenine or 5 mM ethephon or no supplement (control). After this pre-incubation period, the ratio of cells to medium was *ca* 3:1 and the medium became free of adenine, if added. Moreover, adenine addition led after 5 days to considerable intracellular accumulation of xanthosine and related nucleosides, as already reported [4]. Then, 3 g cells was suspended in 1 ml of conditioned medium previously supplied with 4 μCi [U-^{14}C]adenine.

The percentages of incorporation of radioactivity from [^{14}C]adenine 24 hr after feeding are given in Table 1. As expected, cell line E devoid of PA under standard conditions did not show any radioactivity nor UV absorption related to 7-methylxanthine, theobromine, or caffeine, whereas in cell line M more than 31% of the administered radioactivity was found in PA. Apart from this, the incorporation pattern between line E and the caffeine-producing line M was qualitatively similar and differed only quantitatively. In E, more radioactivity from [^{14}C]adenine was incorporated into the purine degradation products, allantoin and uric acid (i.e. 55% versus 33% in M), indicating the prevalence of purine catabolism. Labelling was higher in AMP (15% versus 5% in M) but lower in adenosine (2% versus 10% in M). This observation is in accordance, on one hand, with studies using cell free extracts showing that AMP nucleotidase activity is increased in line M, as compared to E, by a factor of 3 (Fig. 1(a)) and, on the other hand, with cells already harvested after 30 min exhibiting 50 and 10% (E versus M) of radioactivity in AMP and almost exactly the reverse with respect to adenostine radioactivity, i.e. 10% in E and 50% in M. Finally, no other labelled substances than those listed in Table 1 were to be found in this experiment.

When line M cells were pre-incubated for 5 days with 5 mM ethephon prior to [^{14}C]adenine feeding, incorporation into 7-methylxanthine was, as compared to the control, increased from 1% to *ca* 12%, and into total PA from 31% to 49%, respectively (Table 1). On the other hand, labelling of nucleosides (13%) and of purine catabolites (24%) was correspondingly low in ethephon-treated cells, and strong in the control, i.e. 20 and 33%, respectively. In other words, high incorporation into PA (ethephon) goes along with a low incorporation into nucleosides and purine catabolites, and vice versa. Thus, caffeine synthesis is not connected to purine catabolism, but rather competes with it.

After pre-incubation with 1 mM adenine, incorporation of [^{14}C]adenine into total PA was only 11% and purine catabolites were labelled about half as much as in the control. However, most radioactivity (46%; Table 1) was found in the large xanthosine pool extended by the pre-incubation with 1 mM adenine for 5 days (see Fig. 1 in ref. [4]). Apparently, the surplus of adenine is at that time still preferentially diverted into xanthosine, although overall PA formation was already enhanced by pre-incubation with adenine.

Conclusively, neither adenine nor ethephon treatment led to an incorporation of radioactivity from [^{14}C]adenine into [^{14}C]7-methylxanthosine. However, in an additional feeding experiment (data not shown) with a newly established cell-line exhibiting an extremely high percentage of incorporation into PA (between 60 and 80%, depending on the mode of pre-incubation), traces of [^{14}C]7-methylxanthosine (less than 1%) were found after pre-incubation with adenine.

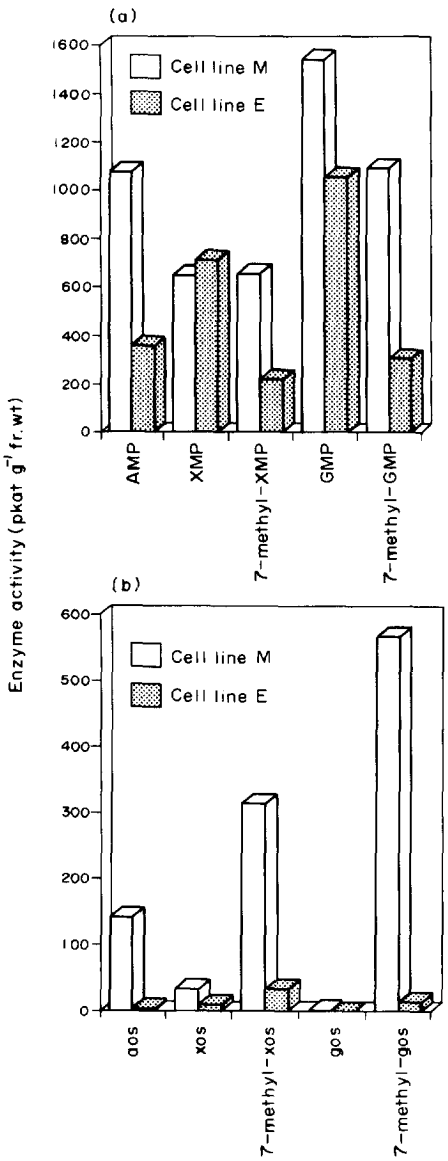


Fig. 1. Nucleotidase (a) and nucleosidase (b) activities in suspension-cultured cells of *Coffea arabica*. Cells of either line M (PA-producing) or line E (devoid of PA production) were grown under experimental control conditions, i.e. without additions, and harvested after 5 days. Enzyme activities were assayed as outlined in the 'Experimental' and using various substrates as indicated on the abscissa. Aos = adenosine, Xos = xanthosine, Gos = guanosine.

Specific radioactivity. We should emphasize the fact, that the *in situ* formation of measurable amounts of [^{14}C]xanthosine (as well as [^{14}C]guanosine) from

[^{14}C]adenine seems to be restricted to cell culture systems and has never been shown in tea seedlings [5], tea flowers and buds [6, 12], tea and coffee leaves [7, 13], tea shoots [9], maté leaves [8] and coffee fruits [11]. Pre-incubation with 1 mM adenine prior to [^{14}C]adenine feeding expanded the cellular pools of these nucleosides and brought about well-measurable UV signals. This unique feature of our suspension-cultured cells is most likely the result of nucleotidase activities being generally much higher (AMP × 8; XMP × 20; GMP × 272) than the corresponding nucleosidase activities (Fig. 1(a) and (b)), and allowed us to compare specific radioactivities along the postulated caffeine pathway.

In a biosynthetic pathway, increasing specific radioactivity of the precursor is always higher than that of the immediate product and reaches the higher maximum earlier. In fact, this was true for the sequence 7-methylxanthine/theobromine/caffeine in both the control and after adenine pre-incubation (Fig. 2(a) and (b) respectively). However, the specific activities of xanthosine and 7-methylxanthine were not in accordance with this principle; in control cells (Fig. 3(a)), 7-methylxanthine showed a higher specific maximum than the putative precursor xanthosine. After pre-incubation with adenine leading to an expanded xanthosine pool, the specific activity of xanthosine was drastically lowered (Fig. 3(b)) and, thus, did not fit into a metabolic precursor sequence

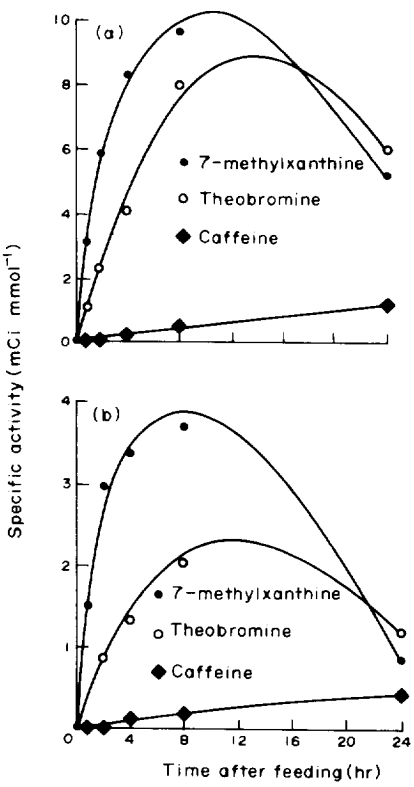


Fig. 2. Specific activity of 7-methylxanthine, theobromine and caffeine after feeding 4 μCi [U-^{14}C]adenine (270 mCi mmol^{-1}) to 3 g cells pre-incubated for 5 days either with 1 mM (b) or without (a, control) adenine.

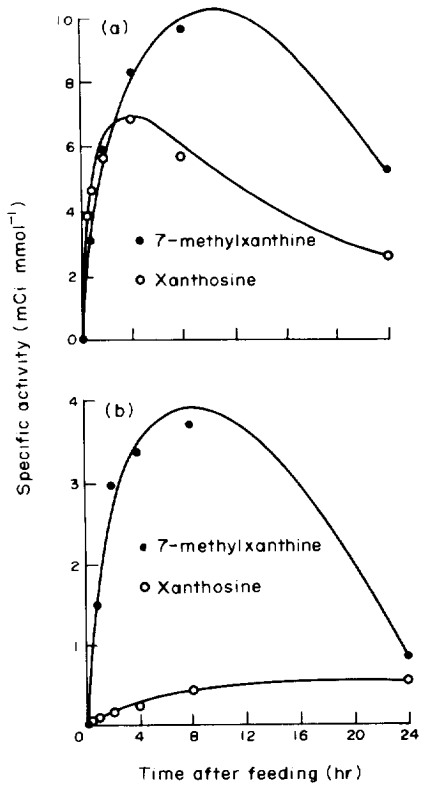


Fig. 3. Specific activity of 7-methylxanthine and xanthosine after feeding 4 μCi [U-^{14}C]adenine (270 mCi mmol^{-1}) to 3 g cells pre-incubated for 5 days either with 1 mM (b) or without (a, control) adenine.

at all. It should be noted that the moderate difference in specific activity between Fig. 2(a) and (b) is due to pre-incubation with 'cold' adenine increasing the size of PA pools. Hence, the kinetic analyses clearly demonstrate that the precursor role of xanthosine in caffeine biosynthesis can be kept up only if we assume the existence of two separate xanthosine pools (see below). Based on the incorporation kinetics and specific activity after pre-incubation with ('cold') adenine, only AMP, adenosine and MTA theoretically fulfill the 'precursor requirements', whereas guanosine, as well as inosine and 7-glucopyranosyladenine [4, 14], must be excluded (data not shown). Due to the limited sensitivity of HPLC, concentration as well as specific activity of labelled nucleotides, such as SAM, XMP, IMP, GMP or pyridine nucleotides, could not be determined.

Studies with [^{14}C]*methionine*

A very selective approach to trace the existence of 7-methylxanthosine is to label its methyl group. The HPLC pattern of radioactive products will be, as compared to ring-labelling, less complex and the search for other methylated products simplified. Therefore, cells were fed with L-[methyl-^{14}C]methionine (45 mCi mmol^{-1}, 22.2 μM). After 90 min, methionine was completely taken up. After 24 hr, more than 34% of the

radioactivity was incorporated into PA (Table 2). The rest must be attributed to [^{14}C]methionine and to its oxidation product [^{14}C]methionine sulphoxide, as based on their retention times. At the first sampling point (1 hr), SAM and MTA (methylthioadenosine) were also labelled by 2.9 and 1.2%, respectively. However, neither [^{14}C]7-methylxanthosine nor any other possible methyl-labelled precursor of 7-methylxanthine could be detected during this experiment.

Subsequently, to achieve maximum precursor accumulation, experimental conditions were arranged as follows. Pre-incubation with (a) 5 mM ethephon or (b) with 1 mM adenine similar to the feeding experiments with [^{14}C]adenine; (c) feed-back inhibition by the addition of 5 mM caffeine 24 hr before tracer feeding; combinations (a + b), (a + c) and (a + b + c). Moreover, to obtain extracts as concentrated as possible, the entire culture (3 g cells and 1 ml medium) was harvested and extracted (with 80% MeOH) at once 2 hr after [^{14}C]methionine feeding, i.e. when incorporation into 7-methylxanthine was expected to be high (Table 2). After drying, the residue was dissolved in 500 μl H_2O and analysed by HPLC and on-line radioactivity detection. Then, the extracts were monitored not only for [^{14}C]7-methylxanthosine but also for other [^{14}C]methylated products, such as 7-methyl-XMP, 7-methyl-IMP, 7-methyl-GMP, 7-methylguanosine and 7-methylinosine. Moreover, aliquots of the extracts were also hydrolysed with 1 N HCl prior to HPLC analyses.

In conclusion, no labelled *N*7-monomethylated products other than 7-methylxanthine or 7-methylxanthosine were detected. The latter occurred only in traces (less than 1%) and exclusively after simultaneous pre-incubation with adenine and ethephon (a + b).

Must xanthosine and 7-methylxanthosine be excluded as caffeine precursors?

The metabolic link of caffeine biosynthesis to primary metabolism consisting of the methylation of xanthosine to 7-methylxanthosine, as postulated by Negishi *et al.* [1–3], could not be clearly confirmed by our investigations. Two important facts are in contradiction to it. Firstly, the time-course of the specific activity of xanthosine after [^{14}C]adenine feeding is not typical for a 7-methylxanthine precursor (Fig. 3(a)), markedly so when the endogeneous xanthosine pool was extended by pre-incubation with adenine prior to tracer administration (Fig. 3(b)). Secondly, the *in situ* formation of [^{14}C]7-methylxanthosine from [U-^{14}C]adenine and L-[methyl-^{14}C]methionine, respectively, was generally absent, even after drastic stimulation of caffeine biosynthesis by various means. Traces of [^{14}C]7-methylxanthosine were detected only under special conditions, i.e. in cultures pre-incubated with 1 mM adenine (xanthosine pool expansion) and simultaneously exhibiting a particularly high PA production either induced by 5 mM ethephon or inherent to the selected cell line. Thus, *in situ* formation of [^{14}C]7-methylxanthosine was dependent on an 'artificially' (by 1 mM adenine) expanded pool of xanthosine either strongly labelled as in the [^{14}C]adenine experiment or unlabelled as in the study with [methyl-^{14}C]methionine.

Table 2. Percent incorporation of L-[methyl-^{14}C]methionine into methylated compounds by cell-line M of *Coffea arabica* after 1, 2, 8 and 24 hr

Time after feeding	1 hr (%)	2 hr (%)	8 hr (%)	24 hr (%)
SAM	2.91	1.00	0	0
MTA	1.15	0.42	0	0
7-Methylxanthine	2.97	5.91	1.88	1.85
Theobromine	3.87	8.51	11.29	5.37
Caffeine	3.09	7.07	12.14	26.85

Cells (3 g) of the caffeine-producing coffee cell-line (M), precultured for 5 days, were fed with 4 μCi L-[methyl-^{14}C]methionine dissolved in 1 ml medium. After 1, 2, 8 and 24 hr, cells were harvested, extracted and analysed by HPLC with on-line radiodetection.

As a consequence, the early reactions in caffeine biosynthesis as proposed and reported in the literature (see above) would be correct only with the following assumptions.

(1) Synthesis of 7-methylxanthosine is metabolically channelled (i.e. by a membrane-bound enzyme system or by a multienzyme complex) and, therefore, after being formed it is not released but immediately transformed to 7-methylxanthine. This would explain the scarce presence of 7-methylxanthosine in the extracts analysed in our studies. Channelling of intermediates is well known in the pathway of other secondary compounds, such as cyanogenic glycosides and hydroxycinnamic acids [15].

(2) There are two endogeneous pools of xanthosine, one acting as a (presumably channelled) precursor pool of PA biosynthesis, the other as a collecting pool for a surplus of purines. This would account for the specific radioactivity of xanthosine being, after feeding [^{14}C]adenine, consistently lower than that of 7-methylxanthine, and additionally lowered by adenine pre-incubation.

Nucleosides are collecting pools for a surplus of adenine and are used for long-term caffeine synthesis

During our studies with suspension-cultured cells, we have experienced adenine effects by three different experimental sets: (a) cells were cultured during 7 days in the presence of 1 mM 'cold' adenine; by this, PA formation was enhanced during the same period and nucleoside pools were drastically increased [4]. *N*-Methyltransferase activity towards 7-methylxanthine was enhanced 2.5-fold (data not shown); (b) cells were fed with [^{14}C]adenine followed after 1 hr by a chase of 0.4 mM adenine; incorporation was, as compared to the pulse alone, lower into PA and higher into nucleosides; (c) cells were pre-incubated for 5 days with 1 mM adenine prior to feeding [^{14}C]adenine. The adenine treatment led to enlarged nucleoside pools, and again, labelling of PA was

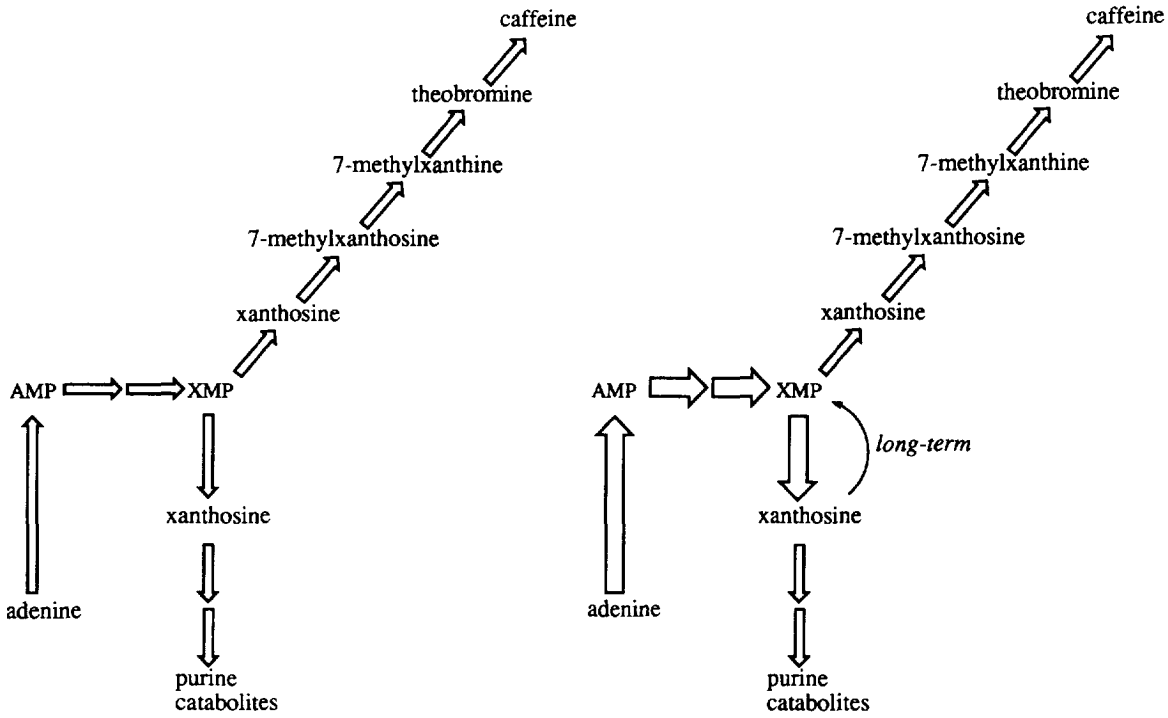
lower and that of nucleosides higher than in the control without adenine preincubation.

Obviously, a surplus of purine moiety originating from adenine cannot adequately enter, on a short-term basis, the caffeine pathway and is diverted into the nucleoside pools. However, a large fraction of the overflow collected in the nucleoside pools, mainly xanthosine, is, in the long term, redirected into caffeine biosynthesis. We suppose that nucleotidase activities diverting excess purine moiety into the nucleoside pools are intrinsically present to some extent, otherwise the chase would not have been effective in rapidly pushing the radioactivity into the nucleoside fractions. Pre-incubation with adenine may, however, inductionally increase nucleotidase activity. Based on the long-term effect of adenine on PA formation we must assume that, by substrate induction not only the crucial enzyme activity mediating the onset of PA synthesis and those activities completing it are increased, but also those redirecting the nucleoside into the caffeine pathway.

In Scheme 1, the two different metabolic situations are depicted and presented in a simplified manner. Without 'cold' adenine added (on the left) a comparatively large fraction of adenine radioactivity will enter the PA pathway. Alternatively, by addition of exogenous adenine (on the right) most of the radioactivity will get into the collecting pool of xanthosine because of highly induced nucleotidase, and a correspondingly small fraction will enter the PA pathway even if overall PA formation may have been increased by pre-incubation with cold adenine as well. In the long term, xanthosine will be re-used for caffeine synthesis.

As outlined above, radioactivity from adenine appeared not only in xanthosine but to some extent in the other nucleoside pools which were also expanded by the adenine treatment. There is no doubt that these interconversions firstly took place at the nucleotide level, since direct transformation of nucleosides has been shown to be limited to that of guanosine to xanthosine by guanosine deaminase [16, 17], which may contribute to xanthosine formation in the collecting pool. Negishi *et al.* [16] have detected nucleoside phosphotransferase activity for inosine, xanthosine and guanosine, and kinase activity for adenosine. In the long term, these activities may divert the nucleosides into the corresponding nucleotides which finally will enter the caffeine pathway, as shown in Scheme 1 for xanthosine yielding XMP. This view may also explain equal incorporation of radioactivity into PA from adenosine, inosine, guanosine and xanthosine as demonstrated by Negishi *et al.* [16].

To summarize, [^{14}C]adenine supplied to a PA-producing tissue is readily activated by the salvage pathway giving adenine nucleotides. After a few and unknown steps, an extremely high fraction of radioactivity is directed into the caffeine pathway. The essential question remains as to the crucial enzymatic reaction diverting the purine moiety into the metabolic channel. Clearly, the first methylation has to be channelled, since under 'normal' metabolic conditions no N7-monomethylated product other than 7-methylxanthine was to be found. Furthermore, methylation of nucleotides, such as XMP or GMP, cannot be ruled out and should be taken into consideration, since nucleotidase activity against 7-methyl-GMP and 7-methyl-XMP (Fig. 1(a)) was consider-



Scheme 1. Metabolic situations in caffeine biosynthesis under 'normal' conditions (left), or with exogenous adenine (right).

ably higher in the caffeine-producing cell-line than in the mutant line without caffeine production.

Finally, we hypothesize that the first methyl group may be introduced at the nucleotide level, most likely into XMP, and that the resulting product, 7-methyl-XMP, was not detected in the related *in vitro* studies [18, 19] because of high nucleotidase activity. In this case, xanthosine would not be a precursor in caffeine biosynthesis. However, further experiments will be required to prove or disprove this hypothesis.

EXPERIMENTAL

Chemicals. Radiochemicals were purchased from Amersham ([U-^{14}C]adenine, 270 mCi mmol^{-1}), CEA ([8-^{14}C]adenine, 49 mCi mmol^{-1}) and Du Pont NEN (L-[methyl-^{14}C]methionine, 45 mCi mmol^{-1}). Nucleotides, 7-methyl-GMP, N7-methylated nucleosides, purine bases, NAD, NADP, SAM, MTA, allantoin, uric acid and ethephon were from Sigma, methylxanthines, as well as nucleosides, from Fluka. 7-Methyl-XMP and 7-methyl-IMP were synthesized in our laboratory by Dr P. Morath according to ref. [20]. 7-Glucopyranosyladenine was isolated as published previously [14].

Suspension-cultured cells. These were established from orthotropic shoots of *Coffea arabica* L. var. *catuai* [21] and grown in commercially available Murashige and Skoog (MS) medium (4.71 g l^{-1}; Flow Laboratories), supplemented with (mg l^{-1}) sucrose (30000), L-cysteine (10), thiamine (1), 2,4-D (1) and kinetin (0.2). The pH was adjusted to 5.7–5.8 with 1 N KOH. Cells (10 g) were usually subcultured at 2-week intervals into 60 ml medium and kept in a 250 ml flask on a gyratory shaker (90 rpm) at 27° in the dark.

Pre-incubation and tracer feeding. Cells (10 g wet wt) in stationary phase were cultured in 10 ml of either medium (= preculturing) or medium containing adenine or ethephon (= pre-incubation) according to ref. [4]. After 5 days, the ratio of cells to medium was 3:1 (w/w). Then, the cells were sepd from the medium by sieving, and to continue with the same ratio (cells to medium) tracer application was as follows. Conditioned medium (1 ml) was transferred into a 25 ml flask and mixed with 4 μCi of either [U-^{14}C]adenine or L-[methyl-^{14}C]methionine. Thereafter, 3 g cells was added and the culture put into the dark on a gyratory shaker (90 rpm). At intervals, samples of the medium (100 μl) and cells (500 mg) were taken aseptically. The medium was filtered through a 0.45 μm filter (Millipore) and directly used for HPLC analysis; cells were extracted prior to analysis.

To assess optimum preculture time, 3 g cells was incubated in 3 ml fr. medium. After 1, 5 or 9 days, respectively, 3 μCi of [U-^{14}C]adenine was directly added and cells extracted after 24 hr of exposure to the tracer. Incorporation into PA was highest (31%) at the 5-day preincubation and recovery of radioactivity was nearly 100%. The percentage recovered but not attributable to PA was exclusively found in various purines, such as nucleosides and nucleotides. Therefore, the 5-day preculture was used in future studies.

Extraction. Cells (500 mg, wet wt) were extracted with 2 ml boiling MeOH (to give *ca* 80% MeOH) according to ref. [4]. The extract was dried and, for HPLC analysis, dissolved in 500 μl H_2O. This vol. corresponds to that of the original cell sample volume. To improve detection in some of the expts with labelled methionine, extracts enriched by a factor of 6 were prepd as follows. The entire culture (3 g cells + 1 ml medium) was extracted in 16 ml boiling MeOH. After sonication (5 min), cells were filtered and washed ×2 with 5 ml MeOH. Filtrates were combined, evapd and the residue dissolved in 500 μl H_2O.

Hydrolysis. An aliquot of the cell extract (100 μl) was mixed with 100 μl 2 N HCl and hydrolysed in a sealed test tube for 1 hr at 100°. Then, to remove the solvent, the sample was dried under a stream of N_2 at 60°. Finally, the residue was dissolved for HPLC analysis in 100 μl H_2O.

Enzymatic studies. Cells (5 g) grown under experimental (control) conditions, i.e. started at a ratio of 1:1 (cell to medium vol.), were harvested after 5 days and homogenized in a pestle and mortar under liquid N_2 together with 1 g PVPP (Sigma) previously equilibrated with buffer (pH 7.3): 0.1 M Tris-HCl, 5 mM mercaptoethanol, 5 mM EDTA and 0.5% ascorbate. The homogenate was transferred into a centrifuge tube and 10 ml buffer was added. After thawing, the suspension was centrifuged at 4° and 20000 *g* for 20 min. From the supernatant (10 ml), proteins were precipitated by slowly adding solid $(NH_4)_2SO_4$ (70% satn) at 4°. The ppt. was collected by centrifugation at 4° and 20000 *g* for 20 min, dissolved in 2.5 ml buffer and the resulting crude extract desalted by centrifugation on Sephadex G-25 (Pharmacia) according to ref. [22]. The column (1 × 4 cm, in a plastic syringe barrel), previously equilibrated with diluted (1:10) buffer, was charged with 250 μl crude extract, put into a tube and centrifuged at 1400 *g* for 2 min, yielding exactly 250 μl eluate (enzyme extract) not diluted by the desalting procedure. Nucleotidase and nucleosidase activities were assayed using 20 μl enzyme extract, 50 μl 0.5 M Tris-HCl buffer (pH 7.5), 10 μl 10 mM $MgCl_2$ and 20 μl 5 mM substrate. Controls were run without enzyme source. Incubation at 30° was stopped after 30 min (nucleotidases) or 60 min (nucleosidases) by immersing the reaction vial for 45 sec into a silicone oil bath at 150°. As tested in separate expts for 7-methyl-XMP, this treatment had a negligible effect on substrate degradation and was superior to protein precipitation by HCl or $HClO_4$. The protein ppt. was removed by centrifugation and the substrate concn in the supernatant was directly measured by HPLC.

HPLC analysis and radiodetection. This was achieved using a liquid chromatograph equipped with a diode-array detector and connected to a radiodetector (Flo-one, Canberra Packard). Separation was performed on a Nucleosil-100 C18 column (5 μm; 4 × 250 mm; precolumn 4 × 20 mm) with 50 mM ammonium phosphate pH 3.8 [A] and MeOH–MeCN (1:1) [B] at a total flow of 1 ml min^{-1} using the following gradient (% B over A): 0–5 min (0); 5–14 min (0–3); 14–25 min (3–20); 25–30 min (20). Column temp. was 40° and inj. vol. 25 μl. Radioac-

tivity was determined on-line in the effluent using 1 ml min^{-1} scintillation liquid. Peaks were identified by comparing UV spectra (library established under separating conditions) and R_ts of authentic standards. The R_ts (min) of compounds relevant to this study were: allantoin (2.4), 7-methyl-IMP (2.6), ATP (3.8), ADP (4.3), SAM (4.4), 7-methyl-XMP (4.6), GMP (5.0), IMP (5.3), 7-methylinosine (5.6), uric acid (5.9), XMP (6.3), NADP (7.0), 7-methyl-GMP (7.0), AMP (7.8), adenine (9.3), 7-methylxanthosine (10.7), 7-glucosyladenine (11.2), 7-methylguanosine (12.0), NAD (12.8), inosine (13.9), guanosine (14.8), 7-methylxanthine (15.9), xanthosine (17.8), adenosine (20.2), theobromine (22.1), MTA (27.5) and caffeine (27.9).

Acknowledgement—We acknowledge the financial assistance of the 'Kommission zur Förderung der wissenschaftlichen Forschung', KWF project Nr. 2038.1.

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