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STIMULATION OF CAFFEINE BIOSYNTHESIS IN SUSPENSION-CULTURED COFFEE CELLS AND THE *IN SITU* EXISTENCE OF 7-METHYLXANTHOSINE

BRIGITTE H. SCHULTHESS and THOMAS W. BAUMANN*

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

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Key Word Index—*Coffea arabica*; Rubiaceae; adenine; caffeine biosynthesis; ethephon; 7-methylxanthosine; purine alkaloids; purine metabolism; tissue culture.

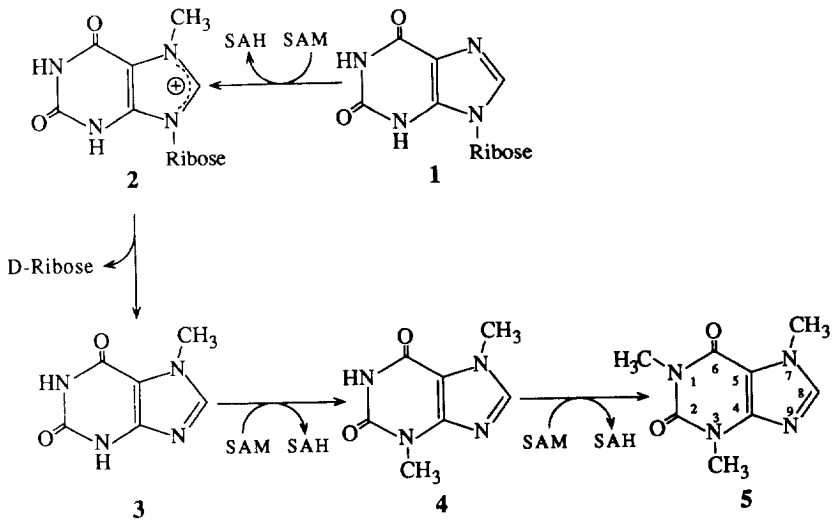
Abstract—Suspension-cultured cells of coffee were subjected to various conditions such as photoperiod, 1 mM adenine, 0.1–10 mM ethephon, or to the combination of both adenine and ethephon. Concentrations of purine bases, nucleosides, nucleotides as well as of purine alkaloids (PA; i.e. 7-methylxanthine, theobromine and caffeine) were measured by HPLC. In the dark, both adenine and ethephon drastically stimulated overall PA formation by a factor of 4 and 7, respectively. Their simultaneous application resulted in an additional increase yielding a stimulation factor of 11. In photoperiod, caffeine formation was, as compared to the control in the dark, enhanced by a factor of 21 without affecting theobromine and 7-methylxanthine pools; additional stimulation by ethephon was not possible. Conversely to light and ethephon, which had no effect on the accumulation of primary purine metabolites, adenine feeding resulted in persistently enlarged pools of nucleosides (xanthosine, guanosine, inosine) and 7-glucopyranosyladenine. 7-Methylxanthosine, the postulated precursor of 7-methylxanthine in caffeine biosynthesis could not be detected under any conditions at any time. Since no other methylated purine was found, it is not yet feasible to discard the '7-methylxanthosine hypothesis'. However, the present investigation provides a suitable system to study the early steps of caffeine biosynthesis by means of radiolabelling kinetics.

INTRODUCTION

The biosynthetic pathway leading from primary metabolism to caffeine is considered to start with a first methylation of xanthosine (**1**) yielding 7-methylxanthosine (**2**). After de-ribosylation the resulting 7-methylxanthine (**3**) is further methylated to theobromine (**4**) and finally to caffeine (**5**) with *S*-adenosylmethionine (SAM) as the source of all three methyl groups (reviewed in ref. [1]). This biosynthetic chain, first postulated by Negishi *et al.* [2–5], has been cited by many authors [6–10] and astonishingly enough has, with one exception [11, 12], never been questioned. In fact, it clearly contradicts earlier studies with cell free extracts from tea leaves [13] and coffee fruits [14] showing xanthosine not to be an acceptor for the methyl group of SAM. Moreover, in leaf discs of coffee simultaneously infiltrated with [methyl-^{14}C]methionine and xanthosine, the latter was not a substrate for methylation [15]. Furthermore, Negishi and co-workers were the only ones who detected, after two-dimensional PC, the *in vivo* formation of 7-methylxanthosine [2, 4, 16]. All other research groups working on caffeine biosynthesis were not able to trace this postulated intermediate, even after feeding [^{14}C]adenine or [methyl-^{14}C]methionine, two well-known efficient precursors in caffeine biosynthesis [6, 8–10, 17–19]. Conversely, ring-labelled 7-methylxanthosine infiltrated into coffee leaves still attached to the tree was efficiently (26%) transformed to caffeine [20]. However, it is conceivable that during uptake unspecific nucleoside hydrolase activity yielded 7-methylxanthine, which in turn was methylated to caffeine. Hence, the biosynthetic pathway leading from xanthosine to caffeine still needs additional proof, and investigations with advanced analytical techniques allowing sensitive detection of hypothetical intermediates are required.

The aim of the present work was to rigorously examine the *in vivo* formation of 7-methylxanthosine ('7-methylxanthosine hypothesis') by selective enhancement of caffeine biosynthesis using the purine alkaloid-producing suspension-cultured cells of *Coffea arabica*. This was targeted by supplying the cells with a high concentration of 'cold' adenine, the most effective precursor among the purine bases as verified for tea [17] and coffee [19]. Adenine is rapidly taken up by cultured cells even at high concentrations and will expectedly increase the endogeneous pool sizes of caffeine precursors, and thus facilitate their detection. Additionally, caffeine formation was triggered by ethephon (2-chloroethylphosphonic acid), an ethylene-releasing compound known to stimulate formation of secondary metabolites in suspension-cultured cells

*Author to whom correspondence should be addressed.



of coffee as well as of *Thalictrum rugosum* [21]. To unambiguously identify all postulated intermediates in caffeine biosynthesis, we established an HPLC system simultaneously separating nucleotides, nucleosides and purine bases.

RESULTS AND DISCUSSION

The experiments were repetitiously carried out with various cell lines of *C. arabica* differing in their capacity to synthesize caffeine and to metabolize adenine. The overall effect of adenine and ethephon on caffeine biosynthesis and purine metabolism was qualitatively the same in all investigations with some minor fluctuations as to time-course and metabolite concentrations. Hence, for each study only one representative experiment with one cell line of the small-aggregate type will be presented.

Stimulation by adenine

Coffee cells were cultured during 7 days in the presence of 1 mM adenine. Within 2 days adenine disappeared from the medium (not shown) and was completely taken up by the cells. Thereafter adenine concentration in the cell extract remained at the low level of the control (around 15 μM), most likely owing to immediate and rapid metabolism after uptake. Main metabolites were xanthosine, inosine, guanosine, 7-glucopyranosyladenine (Fig. 1a) and the three methylated xanthines 7-methylxanthine, theobromine, and caffeine subsequently all together designated as purine alkaloids (PA) (Fig. 2a). Control cell extracts did not contain measurable amounts of inosine or 7-glucopyranosyladenine (for the metabolism of the latter see [22]), and xanthosine as well as guanosine remained below 15 μM (Fig. 1b). According to the suggested pathways of purine metabolism [1], xanthosine and guanosine and most likely also inosine are synthesized from adenosine originating from AMP. However, the pools of both AMP and adenosine (not shown) were not enlarged by the supply of adenine and ranged

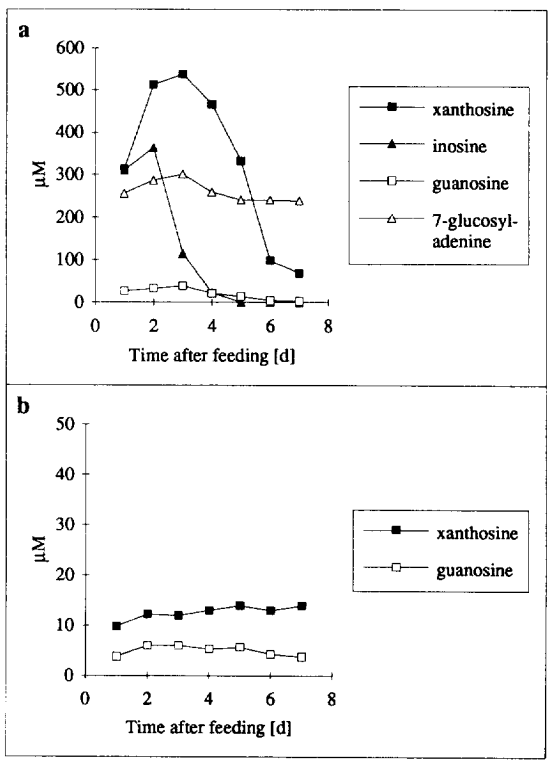


Fig. 1. Formation of purine nucleosides and 7-glucosyladenine in a dark-grown culture (a) as induced by 1 mM adenine or (b) in the control. Control cell extracts (Fig. 1b) did not contain measurable inosine or 7-glucosyladenine, and xanthosine as well as guanosine concentrations were comparatively low (cf. scale).

from 10 to 20 μM and from 15 to 45 μM, respectively, i.e. at the level of the control. Indeed, preliminary tracer studies with the same cell culture system showed, that AMP and adenosine are the main but transient products of [^{14}C]adenine shortly after feeding (over 60% of incorporation into AMP and adenosine after 30 min), this

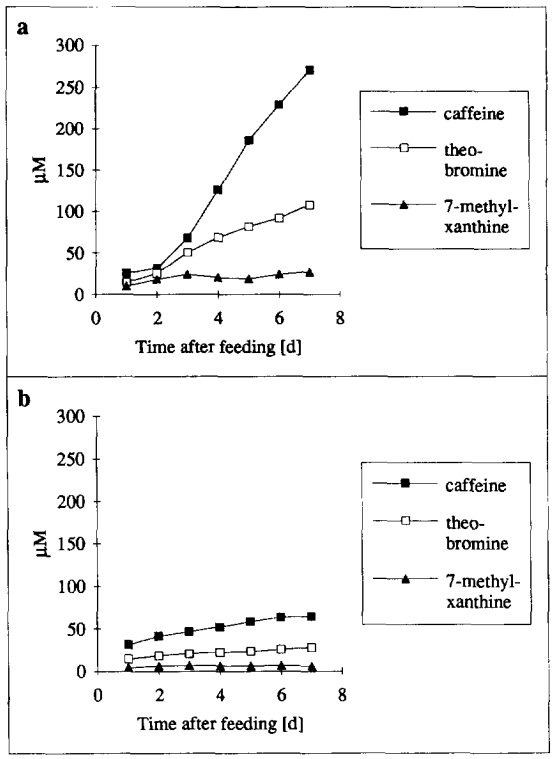


Fig. 2. Purine alkaloid formation in a dark-grown culture (see Fig. 1) as induced by 1 mM adenine (a) or in the control (b).

indicating their rapid turnover. In earlier studies with 1 mM adenine fed to metabolically less active cell lines, we observed a temporary increase of adenosine as well as of *S*-adenosylhomocysteine (SAH) up to 90 and 55 μM, respectively, which coincided with a delay to stimulation of caffeine formation. Thus, this delay must be attributed most likely to methyltransferase inhibition by SAH which may have accumulated as a result of adenosine-mediated inhibition of SAH hydrolase [23]. Similarly, owing to the high metabolic activity of the cell line used in the present experiment, the level of 7-glucopyranosyladenine was, in contrast to our previous report [22], not transiently increased and remained fairly constant through the entire period of observation (Fig. 1a). The pools of ADP, ATP, IMP, GMP and XMP were always very small or not detectable and were not affected within the range of detection.

Although in cell cultures supplied with adenine, PA (Fig. 2a; day 7) and xanthosine (Fig. 1a; day 3) levels were, as compared to the control (Fig. 2b and 1b), increased by a factor of 4 and 45, respectively, the putative precursor 7-methylxanthosine could not be detected at any time point.

Stimulation by ethephon

It should be mentioned that in suspension-cultured cells caffeine is the accumulated end-product of PA biosynthesis [24] and, in contrast to ageing leaves [25], is not further metabolized. In our system using a 1:1 cell to medium ratio, caffeine accumulation rate as well as growth of untreated control cultures starts to decrease after 7 days. However, stimulated cultures then still exhibit a considerable PA formation rate, suggesting the pools of precursors to be of optimum size for their detection. Therefore, cultures were harvested and analysed at this time point.

As compared to the control without ethephon, synthesis of each of 7-methylxanthine, theobromine and caffeine was, after 7 days, distinctly enhanced by increasing concentrations of ethephon up to 5 mM (Fig. 3). At 10 mM ethephon the sum of the three products was about equal to that at 5 mM, but final methylation of theobromine to caffeine was inhibited resulting in a 14- and nine-fold accumulation of 7-methylxanthine and theobromine, respectively. Despite a seven-fold overall stimulation of PA at 5 or 10 mM (Fig. 3), 7-methylxanthosine was not found. We have repeatedly observed that caffeine biosynthesis is rapidly accelerated by ethephon (ethylene) without the size of the various purine pools being affected. Also in the present study these were almost identical in ethephon treated and control cells (see Fig. 1b), the xanthosine pool included.

Combination of adenine with ethephon

When adenine (1 mM) and ethephon (5 mM) were fed simultaneously, an additive stimulatory effect on caffeine synthesis was induced (Fig. 4) reaching, as compared to the control, an overall factor of 11. Ethephon and adenine alone stimulated by a factor of seven and four, respectively. The combination led to a high accumulation of both theobromine and 7-methylxanthine (about 20 × the control) probably owing to a limited capacity of the last methyltransferase reaction, either inherent or effected by product (caffeine) inhibition. One would expect that under this metabolic situation the potential precursor of

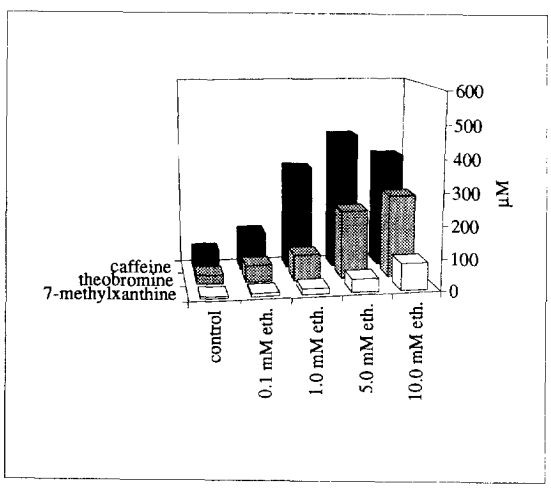


Fig. 3. Purine alkaloid formation in dependence of ethephon concentration. Cells were cultivated in the dark and harvested after 7 days.

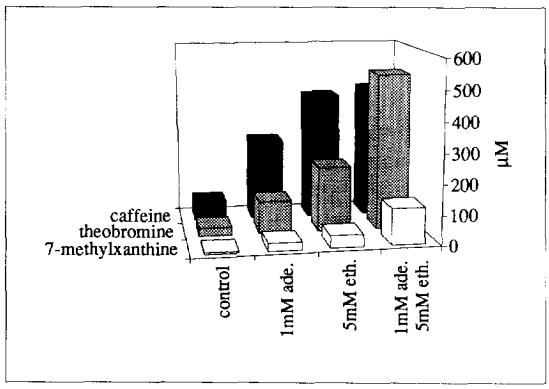


Fig. 4. Purine alkaloid formation as influenced by adenine (1 mM), ethephon (5 mM) or the combination of both. The data for the separate additions of adenine and ethephon were taken from Figs 2 and 3, respectively. Cells were cultivated in the dark and harvested after 7 days.

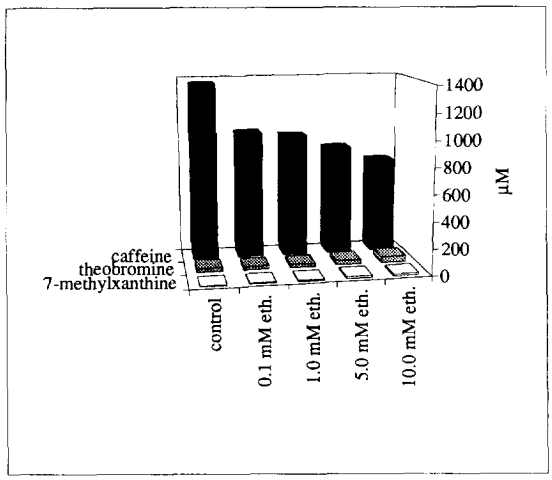


Fig. 5. Purine alkaloid formation in dependence of ethephon concentration. Cells were cultured in photoperiod and harvested after 7 days. The corresponding data for chlorogenic acids are presented in Table 1.

7-methylxanthine, 7-methylxanthosine was to be found. But this was not the case.

Photoperiodic conditions without and with ethephon

In accordance with ref. [26], light *per se* (Fig. 5; control) stimulated, as compared to the control in the dark (Fig. 3), caffeine formation by a factor of about 21 without affecting the pools of theobromine and 7-methylxanthine. An additional enhancement by ethephon did not take place (Fig. 5; 0.1–10 mM ethephon). On the contrary, increasing ethephon concentrations steadily inhibited caffeine synthesis under photoperiodic conditions. Moreover, synthesis of chlorogenic acids known to be responsible for purine alkaloid complexation and compartmentation [27] was inhibited by ethephon as well (Table 1); this in contrast to the dark conditions, where both purine alkaloids (Fig. 3) and, less pronounced, chlorogenic acids (Table 1) were enhanced by increasing concentrations of ethephon. Separate studies with exogeneous caffeine or PAL inhibitors indicated a complex regulatory interdependence between chlorogenic acids and purine alkaloids. In essence, as will be published elsewhere in detail, there is a cell line-specific purine alkaloid concentration, in this case at about 700 μM, which cannot be surpassed without a concomitant drastic synthesis of chlorogenic acids. If massive formation of chlorogenic acids is impaired, deficient caffeine complexation results in the inhibition of the purine alkaloid biosynthesis. High caffeine concentrations as reached under photoperiod (Fig. 5) are always accompanied and governed by high chlorogenic acid concentrations.

Finally, we should mention that in none of the experiments performed in photoperiod was 7-methylxanthosine detectable, and that the pools of adenine metabolites, including xanthosine, remained low under all conditions tested.

Outlook

Both adenine and ethephon were demonstrated to be effective stimulators of caffeine biosynthesis in coffee cell suspension cultures grown in the dark, and resulted in increased levels of 7-methylxanthine, theobromine, and caffeine. Neither ethephon treatment inducing a particularly high accumulation of 7-methylxanthine (**3**), nor cultivation with adenine resulting in considerable formation of xanthosine (**1**), led to detectable accumulation of 7-methylxanthosine (**2**), the postulated methylation product of xanthosine and precursor of 7-methylxanthine. This negative finding is even true for the combined addition of adenine and ethephon.

Hence, we were not able to confirm the *in situ* synthesis of 7-methylxanthosine. On the other side, we could not detect any other methylated purine during the present studies and, therefore, rejecting the '7-methylxanthosine hypothesis' would not be appropriate. Additional investigations with radiolabelled precursors such as [^{14}C]adenine or [methyl-^{14}C]methionine will be required. The prerequisites for successful labelling studies are now established: ethephon treatment will expectedly increase the incorporation rate into the members of the caffeine biosynthesis chain, and addition of unlabelled adenine will expand the pool of xanthosine and of other potential precursors, thus allowing determination of concentration and consequently of specific activity necessary to identify the position of a postulated precursor.

EXPERIMENTAL

Chemicals. Nucleotides, 7-methylxanthosine and ethephon were from Sigma; methylxanthines, nucleosides and 5-CQA from Fluka, Buchs, Switzerland. 3,5-diCQA was kindly provided by Dr Michael N. Clifford, University of

Table 1. Influence of ethephon on the formation of caffeoylquinic acids in coffee cells cultured either in the dark or under photoperiod

Ethephon (mM)	Dark 5-CQA (mM)	Dark 3,5-diCQA (mM)	Dark Σ (mM)	Photoperiod 5-CQA (mM)	Photoperiod 3,5-diCQA (mM)	Photoperiod Σ (mM)
0 (control)	0.1	0.0	0.1	8.4	8.4	16.8
0.1	0.2	0.0	0.2	5.6	5.3	10.9
1.0	0.3	0.3	0.6	4.2	6.1	10.3
5.0	0.3	0.7	1.0	2.8	6.3	9.1
10.0	0.5	0.8	1.2	2.5	4.6	7.1

Cells were cultivated at the ethephon concentration indicated and after 7 days analysed for 5-CQA (5-caffeoylquinic acid) and 3,5-diCQA (3,5-dicaffeoylquinic acid).

Surrey, Guildford, Surrey, U.K. 7-Glucopyranosyladenine was isolated according to ref. [22].

Suspension-cultured cells. Grown in commercially available Murashige and Skoog (MS) medium (4.71 gl^{-1}; Flow Laboratories), supplemented with (mg l^{-1}) sucrose (30 000), 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.

Experimental conditions. Cells (10 g wet wt) in the stationary phase of growth were mixed with 10 ml of commercially available Murashige and Skoog (MS) medium. Since free space (i.e. predominantly medium adhering to the cells) of the wet cell portion is 78% (determined by radioactively labelled mannitol and inulin [22]) and, therefore, nearly halves (56%) the concentration of a compound added to the medium, initial medium concn of a substance fed was doubled. For example, treatment with '1 mM adenine' means that cells (10 g) were mixed with medium (10 ml) containing 2 mM adenine to give the final theoretical concn of 1.12 mM. Adenine was supplied directly into the medium before autoclaving, whereas ethephon (200 μl) was administered as a 200-fold concentrated sterile-filtered soln shortly prior to the addition of the cells. Cultures were kept for 7 days either in the dark (i.e. standard conditions, see above) or under a photoperiod of 13 hr in a growth chamber (26°, 350 $\mu E\ m^{-2}\ sec^{-1}$). At intervals, medium (500 μl) and cell (500 mg) samples were taken aseptically. The medium was filtered through a 0.45 μm filter (Millex-HV 13, Millipore) and directly used for HPLC analysis, whereas cells were extracted (see below) prior to analysis.

Extraction. Freshly harvested cells were immediately extracted in a syringe barrel with the outlet closed from the outside with a plastic cap and covered from the inside with a Whatman GF/F glass filter supported by a porous polyethylene disc. The barrel was filled with 2 ml MeOH, mounted in a H_2O bath at 80°, and the cells (500 mg) were gradually added and extracted for 3 min in the boiling MeOH. Then, the suspension was sonicated for 5 min at room temp. Thereafter the barrel without cap was fixed into a centrifuge tube and the assembly was centrifuged at 4000 *g* for 3 min. The residue was washed ×2 with 2 ml MeOH. The filtrates were combined and dried overnight at room temp. and red. pres. The residue was dissolved in 500 μl H_2O (corresponding in about to the original cell sample volume), and after filtration (Millex-HV 13, 0.45 μm, Millipore) the soln was injected for HPLC analysis. It should be mentioned that metabolite concs in the cell extract, as presented in the result part, are not free space-corrected. Arithmetically, cellular concs are higher by a factor of 4.54 with the exception of purine alkaloids in dark-grown cultures almost evenly distributed between cells and medium.

HPLC separation. Performed on a Nucleosil-100 C18 column (5 μm; 4 × 250 mm; pre column 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} and by the following gradient (% B over A): 0–5 min (0); 5–14 min (0–3); 14–25 min (3–20); 25–35 min (20–70). Parameters were controlled by a Hewlett Packard liquid chromatograph HP 1090 equipped with a diode array detector set at 254 and 323 nm. Column temp. was 40°C and injection vol. 25 μl. Peak identification was achieved by comparing UV spectrum (library established under separating conditions) and retention time of authentic standards. The R_ts (min) were as follows: AMP (7.8), adenine (9.3), 7-methylxanthosine (10.7), inosine (13.9), guanosine (14.8), 7-methylxanthine (15.9), xanthosine (17.8), adenosine (20.2), theobromine (22.1), 5'-CQA (25.0), caffeine (27.9), 3,5-diCCQA (30.9).

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