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Effect of multiple factors on accumulation of nucleosides and bases in *Cordyceps militaris*

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Abstract

In order to improve the accumulation of valuable metabolites (3′-deoxyadenosine, adenosine, guanosine, cytidine, uridine, adenine and uracil) in *Cordyceps militaris*, multiple factors such as carbon sources, nitrogen sources, metal ions and fermentation duration were investigated in liquid shake flasks. Using both potato broth and glucose as carbon sources were found to facilitate the production of nucleosides and bases most, and 2% of glucose was the most suitable initial level. Mixture of 0.3% yeast extract with 0.3% peptone was the best selection of nitrogen sources and supplementing with 0.1 mmol/L Mn^{2+} achieved the maximum biomass and biosynthesis of nucleosides and bases among all investigated metal ions. The most suitable harvest time for *C. militaris* was found to be the 6th day, because the production of 3′-deoxyadenosine, adenosine and uridine began to speed up from the later exponential growth phase until the 6th day. Finally, under optimal culture conditions, the contents of 3′-deoxyadenosine, adenosine, guanosine, cytidine, uridine, adenine and uracil were increased to 0.212 ± 0.014 mg/g, 5.05 ± 0.31 mg/g, 4.03 ± 0.30 mg/g, 0.556 ± 0.029 mg/g, 6.39 ± 0.33 mg/g, 0.208 ± 0.016 mg/g and 0.437 ± 0.027 mg/g, respectively.

Keywords: *Cordyceps militaris*; Nucleoside; Base; Carbon sources; Nitrogen sources; Metal ions

1. Introduction

Medicinal mushroom is an abundant source of useful natural products with biological activities. As one of the important traditional Chinese medicins, *Cordyceps militaris* (an entomopathogenic fungus) which belonging to the class *Ascomycetes*, has been used extensively as a crude drug and a folk tonic food in East Asia (Ying, Mao, Mao, Zong, & Wen, 1987). It contains many kinds of active components (such as mannitol, polysaccharides and cordycepin), and due to its various physiological activities it is now used for multiple medicinal purposes (Song, Jeon, Yang, Ra, & Sung, 1998).

Nucleosides and bases (such as 3′-deoxyadenosine, adenosine, guanosine, cytidine, uridine, adenine and uracil) are some of the main active components in *C. militaris*, and they are commonly considered chemical markers for quality control of *Cordyceps* (Pharmacopoica Commission of PRC, 2000; Zheng, Dong, & She, 1999). 3′-Deoxyadenosine (cordycepin) is a unique nucleoside analogue in *Cordyceps* sp., and there are many reports concerning its isolation and pharmacological functions. (Cunningham, Hutchinson, Manson, & Spring, 1951; Kodama, McCaffrey, Yusa, & Mitsuya, 2000; Melling, Belton, Kitching, & Stones, 1972; Seldin, Urbano, McCaffrey, & Fross, 1997). Adenosine has many pharmacological effects (Pelleg & Porter, 1990), it can be used as a cardioprotective and therapeutic agent for chronic heart failure (Kitakaze & Hori, 2000), and it can inhibit the release of neurotransmitters in the central nervous system (Ribeizo, 1995). The other nucleosides and bases can also be used to treat many different diseases (Carlezon, Mague, Parow, Stoll, & Cohen, 2005; Carlezon, Pliakas, Parow, Detke, & Cohen,

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2002; Connolly & Duley, 1999; Regina, Prato, & Emilio, 2003). Therefore, the quantities of these nucleosides and bases are very important to evaluate the quality of *C. militaris*.

Due to the requirements of specific hosts and strict growth environments,*C. militaris* is very scarce in nature; hence, its submerged cultivation is viewed as a promising field. Moreover, some experiments haven proven that the chemical components of natural and cultured *C. militaris* are similar (Jiang & Sun, 1999; Tong, Kuang, Wu, Zhang, & Ren, 1997).

Carbon sources, nitrogen sources, metal ions and duration of fermentation are directly linked with cell proliferation and metabolite biosynthesis; thus, the work was focused on these multiple aspects on the growth of submerged cultured *C. militaris* and the accumulation of 3′-deoxyadenosine, adenosine, guanosine, cytidine, uridine, adenine and uracil. Since many pharmacological effects of traditional Chinese medicines come from synergetic actions of several bioactive components, the culture medium was optimized comprehensively based on the contents of all these seven valuable nucleosides and bases.

2. Material and methods

2.1. Materials

Natural *Cordyceps militaris* was collected from Sichuan, China.

2.2. Sample preparations

The isolated strain (*Cordyceps militaris*) was initially maintained on potato dextrose agar slants (contains 20% potato broth, 0.5% glucose, 0.5% yeast extract, 0.05% $MgCl_2$, 0.1% KH_2PO_4, 2% agarose). It was inoculated into 250 ml seed flasks containing 100 ml liquid substrate (the same as above, only without agarose) and grew on a rotary shaker incubator at 160 r/min for 5 days at 23 °C. Then it was inoculated at the final concentration of 5% into 500 ml flasks containing 200 ml medium and was cultured under the same conditions for 5 days. The productions were centrifuged at 4000*g* for 15 min, the residual mycelium were dried to constant weight and homogenized to powders.

About 0.5 g of sample powders was extracted with 50 ml 20% ethanol by ultrasonic cell crushing, then centrifuged at 10000*g* for 15 min. The residue was re-extracted until the extraction solvents became colourless. The supernatant was combined and vacuum-evaporated on a water bath at 50 °C until dry. The residue was dissolved and transferred into a 10 ml volumetric flask with double distilled water, and then filtered with a 0.45 μm membrane.

2.3. Analysis

Waters high-performance liquid chromatography system consists of automated gradient controller, 510 pump and 486 UV/visible tunable absorbance detector (Waters, Massachusetts, USA). Kromasil C_8 analytical column, 200 mm × 4.6 mm, i.d., 5 μm (Turner, Tianjin, China); detection wavelength, UV 260 nm; working temperature, 30 °C; flow-rate, 1.0 ml/min; injection volume, 20 μl.

The chromatographic conditions were set up as follows: solvent A, double distilled water; solvent B, methanol; linear gradient elution 2/2/15/15%B at 0/10/10.2/25 min. The correlation coefficients of the standard calibration curves of all components were higher than 0.9990. The accuracy and precision of seven nucleosides and bases expressed as RSD were less than 5.04%, the mean recoveries of them were between 95.8% and 102.5%.

Residual sugar concentration was assayed by a phenol–sulphuric acid method (Dubois, Gilles, Hamilton, & Rebers, 1956). Residual ammoniacal nitrogen concentration was estimated by Cai and Yuan's method (1982).

2.4. Effect of multiple factors on biomass and accumulation of nucleosides and bases in C. militaris

2.4.1. Different carbon sources

The investigated sole carbon sources included 20% (v/v) potato broth, 4% (w/v) glucose, sucrose and maltose, respectively. The complex carbon sources used 20% potato broth as basic carbon sources, and then supplemented with 2% glucose, sucrose and maltose, respectively (the abbreviations of these groups were PG, PS and PM, respectively). The nitrogen sources were 0.5% yeast extract, and the medium also contained 0.05% $MgCl_2$ and 0.1% KH_2PO_4.

2.4.2. Different initial glucose concentrations

The basic carbon source was 20% potato broth, and then supplemented with different initial concentrations of glucose (0.5, 1, 2, 3 and 6%).

2.4.3. Different nitrogen sources

The tested nitrogen sources included 0.1%, 0.3%, 0.5% yeast extract, peptone and combination of yeast extract and peptone, respectively.

2.4.4. Different metal ions

The investigated metal ions contained 0.01 mmol/l, 0.05 mmol/l, 0.1 mmol/l, 0.2 mmol/l, 0.5 mmol/l Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+} and Li^{+}, respectively. The medium without these meal ions was set as the control group.

2.4.5. Different harvest time

The cultured *C. militaris* was harvested every 24 h until 7th day, but the determination of nucleosides and bases started from 48th hour.

2.5. Statistical analysis

All values are means of three replicates ±SD. For multiple comparisons, the Tukey test was used for statistical significance analyses at $P < 0.05$.

3. Results

3.1. Effect of different carbon sources

Carbohydrates are important carbon and energy sources for cells, so in an attempt to identify a suitable substrate for *C. militaris* growth, nucleosides and bases production, several carbon sources were investigated. As shown in Fig. 1 and Table 1, compared with sugar groups, potato broth was not propitious to be utilized, as it drastically suppressed cell growth (9.6 ± 1.8 g at day 5). The kinetics of residual sugar concentration also supported the conclusion since the carbon sources were slowly consumed by *C. militaris*. When using 4% of sugars, the biomass rapidly increased with a corresponding sharp decrease in residual sugars (the unlisted results of maltose and sucrose were similar with the glucose). However, the productions of most components in sugar groups were low.

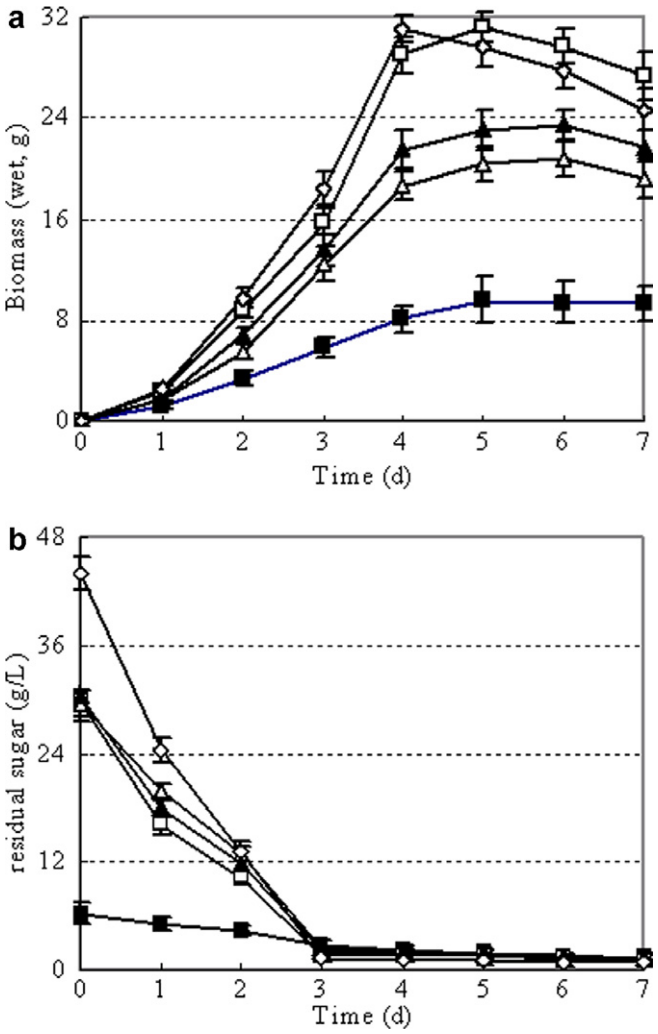

Fig. 1. The biomass (a) and residual sugar concentrations (b) of different carbon sources during the submerged cultivation of *C. militaris*. (■) 20% potato broth; (◇) 4% glucose; (□) 20% potato broth with 2% sucrose; (▲) 20% potato broth with 2% glucose; (△) 20% potato broth with 2% maltose. The error bars in the figure indicated the standard derivations from three independent samples.

All tested complex carbon sources supported cell growth better than potato broth (from 20.4 ± 1.3 g to 29.6 ± 1.4 g at day 5, shown in Fig. 1), and a maximal cell growth was obtained in PS group (20% potato broth supplemented with 2% sucrose), this was in concordance with Park, Kim, Hwang and Yun's report (2001). However, the production of nucleosides and bases was low (Table 1). The biomass of PG (20% potato broth supplemented with 2% glucose) was lower than PS, but it was the most suitable carbon sources for accumulation of nucleosides and bases.

3.2. Effect of different initial glucose concentrations

Based on the results above, potato broth with glucose was selected as the best carbon source. Further study concentrated on the best initial concentration of glucose. As shown in Table 2, with the increased concentration from 0.5% to 6%, the biomass increased proportionately from 20.2 ± 1.5 g to 31.2 ± 1.4 g; however, the contents of nucleosides and bases did not follow the same trend, as maximum quantities of adenosine, guanosine, cytidine, adenine and uracil were obtained at 2% glucose. Thus, 2% was regarded as the best initial concentration of glucose.

3.3. Effect of nitrogen sources

Other than the carbon source, the nitrogen source was also important factor in biomass and accumulation of metabolites. Unlisted results showed that the effects of peptone, yeast extracts and the two combined at different levels on *C. militaris* growth were minor. Moreover, the time pro-

Table 1
Effect of different carbon sources on accumulation of nucleosides and bases (mg/g) in *C. militaris*

Carbon sources	20% Potato broth	4% Glucose	20% Potato broth with 2% sucrose	20% Potato broth with 2% maltose	20% Potato broth with 2% glucose
Uridine	0.902 ± 0.029a	1.65 ± 0.057c	1.25 ± 0.039b	2.56 ± 0.057c	2.84 ± 0.089c
Adenosine	1.39 ± 0.042a	1.60 ± 0.079c	0.958 ± 0.038b	2.11 ± 0.079c	3.01 ± 0.102d
Guanosine	1.03 ± 0.029a	1.031 ± 0.027a	0.345 ± 0.019b	0.988 ± 0.027a	1.36 ± 0.060c
3′-deoxyadenosine	Trace	0.117 ± 0.011b	0.098 ± 0.009a	0.189 ± 0.011b	0.225 ± 0.015b
Cytidine	0.118 ± 0.029a	0.362 ± 0.014b	0.369 ± 0.013b	0.326 ± 0.014b	0.402 ± 0.017b
Uracil	0.254 ± 0.029a	0.149 ± 0.008b	0.175 ± 0.006b	0.194 ± 0.008b	0.227 ± 0.015ab
Adenine	0.042 ± 0.004a	0.113 ± 0.005b	0.040 ± 0.003a	0.103 ± 0.005b	0.141 ± 0.007c

All values are means of three replicates ±SD. Values with same letters within rows are not significantly different at $P < 0.05$.

Table 2
Effect of initial glucose concentrations on biomass (wet, g) and accumulation of nucleosides and bases (mg/g) in *C. militaris*

Carbon sources	20% Potato broth with 0.5% glucose	20% Potato broth with 1% glucose	20% Potato broth with 2% glucose	20% Potato broth with 3% glucose	20% Potato broth with 6% glucose
Mycelium	20.2 ± 1.5a	23.1 ± 1.5a	29.1 ± 1.2b	30.4 ± 1.1b	31.2 ± 1.4b
Uridine	2.48 ± 0.108a	2.84 ± 0.089ab	3.14 ± 0.117b	3.43 ± 0.119b	3.01 ± 0.131b
Adenosine	2.55 ± 0.130a	3.01 ± 0.102ab	3.59 ± 0.140b	3.35 ± 0.143b	3.07 ± 0.134ab
Guanosine	1.39 ± 0.076a	1.36 ± 0.060a	1.57 ± 0.071a	1.54 ± 0.059a	1.38 ± 0.055a
3′-deoxyadenosine	0.204 ± 0.013a	0.225 ± 0.015a	0.211 ± 0.014a	0.156 ± 0.012b	0.164 ± 0.011b
Cytidine	0.362 ± 0.024a	0.402 ± 0.017a	0.444 ± 0.017b	0.425 ±0.018ab	0.397 ± 0.016a
Uracil	0.202 ± 0.013a	0.227 ± 0.015a	0.352 ± 0.018b	0.328 ± 0.019b	0.288 ± 0.017b
Adenine	0.151 ± 0.009a	0.141 ± 0.007a	0.157 ± 0.011a	0.147 ± 0.012a	0.156 ± 0.010a

All values are means of three replicates ±SD. Values with same letters within rows are not significantly different at $P < 0.05$.

files of residual ammoniacal nitrogen concentrations of them were similar. However, different nitrogen sources showed different effects on nucleosides and bases production though the differences were not noticeable in most cases. Among all, utilizing both yeast extract and peptone was most beneficial to guanosine, adenine, adenosine and 3′-deoxyadenosine biosynthesis. Through further investigations, combination of 0.3% yeast extract and 0.3% peptone was the best selection.

3.4. Effect of metal ions

Metal ions were required for metabolism of organism; to further optimize the medium, the effects of multiple metal ions at different levels were studied. Since cell growth was significantly inhibited by high concentrations of metal ions (0.2 mmol/l and 0.5 mmol/l), the study focused on the concentrations below 0.2 mmol/l. Low levels of metal ions (0.01 mmol/l and 0.05 mmol/l) did not affect the biomass and the production of nucleosides and bases much. However, at 0.1 mmol/l, some metal ions could stimulate the growth and synthesis of some components. Among them, Mn^{2+} showed the best result. The biomass and contents of uridine, guanosine, adenosine and uracil were significantly increased from 29.1 ± 1.8 g, 3.14 ± 0.09 mg/g, 1.43 ± 0.07 mg/g, 3.56 ± 0.010 mg/g and 0.316 ± 0.012 mg/g of the control to 33.1 ± 1.7 g, 6.39 ± 0.20 mg/g, 4.83 ± 0.14 mg/g, 4.96 ± 0.15 mg/g and 0.420 ± 0.015 mg/g of Mn^{2+} group, respectively.

3.5. Effect of harvest time

After the optimal medium was obtained, the most suitable duration of fermentation was investigated. As shown in Fig. 2, *C. militaris* rapidly grew for about 3 days, and the nutrition sources were almost exhausted in this period of time. The maximum yield of the biomass was obtained after about 4 days' fermentation, followed by a stationary phase, then a decline phase. During the latter exponential growth phase and the stationary phase, the residual sugars and ammoniacal nitrogen decreased slowly.

Fig. 3 summarized the time profiles of nucleosides and bases production in *C. militaris*. When entering the decline phase, the mycelium began to autolyze; however, the quantities of most nucleosides and bases did not decrease immediately (only the contents of guanosine slightly decrease), but were maintained over a period of time or even increased.

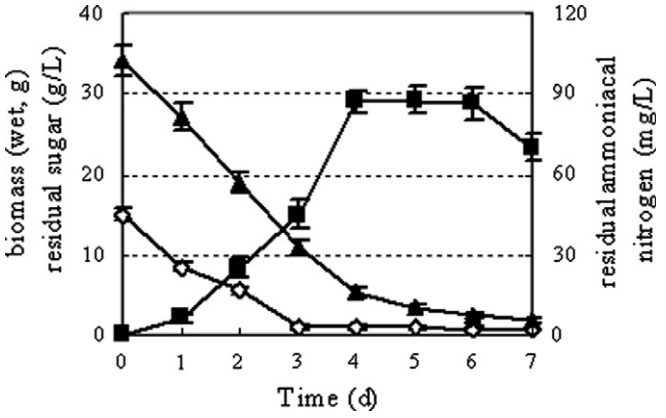

Fig. 2. The biomass, residual sugar and ammoniacal nitrogen concentrations during the submerged cultivation of *C. militaris*. (■) biomass; (◇) residual sugar concentrations; (▲) residual ammoniacal nitrogen concentrations. The error bars in the figure indicated the standard derivations from three independent samples.

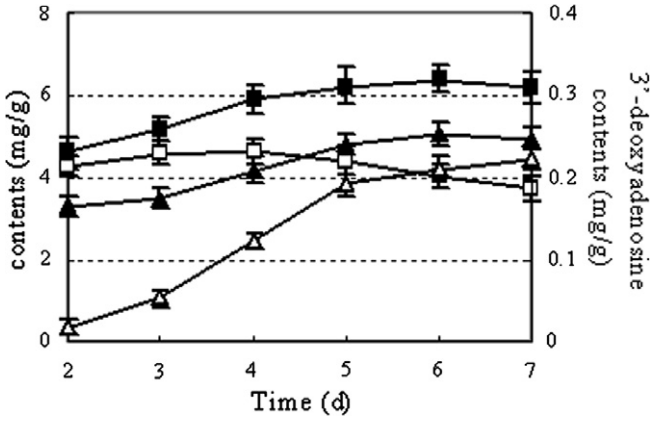

Fig. 3. The accumulation of nucleosides and bases during the submerged cultivation of *C. militaris*. (■) uridine; (□) guanosine; (▲) adenosine; (△) 3′-deoxyadenosine. The time profiles of other three components were not shown since the varieties of their quantities were not significant. ($P > 0.05$) The error bars in the figure indicated the standard derivations from three independent samples.

The content of 3′-deoxyadenosine increased highly from 2nd day to 7th day (from 0.018 ± 0.009 mg/g to 0.223 ± 0.011 mg/g), and the amount of adenosine and uridine also increased from 3.31 ± 0.25 mg/g, 4.61 ± 0.38 mg/g at the 2nd day to 5.05 ± 0.31 mg/g, 6.39 ± 0.33 mg/g at the 6th day, respectively, but their increasing speed was much lower.

4. Discussion

In this work, the effects of carbon sources, nitrogen sources, metal ions and duration of fermentation were studied in order to improve the cell growth and nucleosides and bases accumulation. As shown in Section 3.1, using solely the potato broth was not good for cell growth; it took the cells a long time to produce the corresponding enzymes required to consume the complex carbohydrates. However, potato broth could afford a long period of time for cell growth. The sugars were efficient carbon sources for growth, but it resulted in low accumulation of nucleosides and bases. Thus, it was essential to mix potato broth with a low quantity of sugars as carbon sources to balance growth with synthesis of the nucleosides and bases. As far as we knew, there are few reports that use complex carbon sources to culture *C. militaris*. Results showed that potato broth with glucose (PG) was an optimal carbon source for the synthesis of most components, though potato broth with sucrose (PS) was best for cell growth. The possible reason why different sugars had different effects on metabolites formation was that they might have different effects of catabolic repression on the cellular metabolism. In addition, it could be concluded from the results that the excessively high speed of growth and consuming carbon sources (such as in sugar groups and PS) should be prevented, because it was disadvantage to production of nucleosides and bases. Thus, the cell growing status of PG was found to be most suitable.

Initial concentration of carbon sources was another important factor for growth and metabolites accumulation, and 2% glucose was considered as the best selection. A possible reason of high initial glucose resulted in low contents of nucleosides and bases was osmotic pressure caused by a high glucose concentration. High levels of glucose might be detrimental to the metabolic biosynthesis though cell growth was still promoted; a similar phenomenon was found in ganoderic acid biosynthesis by *Ganoderma lucidum* (Fang & Zhong, 2002). Another possible reason was the carbon catabolite repression caused by glucose as reported previously (Belinchón & Gancedo, 2003).

Section 3.3 revealed that the types and levels of nitrogen sources were inferior to carbon sources for both the cell growth and the yield of metabolites. Among all investigated nitrogen sources, combination of 0.3% yeast extract and 0.3% peptone was found to be the most suitable substrates.

High concentrations of metal ions are poisonous to cells, but suitable levels of certain metal ions are beneficial to cell growth and metabolite synthesis (Yue & Zhong, 2005). In this work, Mn^{2+} was identified to be a recommendable growth, nucleosides and bases production stimulator at 0.1 mmol/l. Wang, Gu, and Yuan (2006) reported that addition of a suitable concentration of Mn^{2+} into the medium could induce the production of antioxidant enzymes. This was presumed to be a partial reason for the improved growth of *C. militaris*, since Mn^{2+} could help the cell overcame the oxidative stress better. The reason why Mn^{2+} was advantageous to synthesize nucleosides and bases was unknown, but it might be a necessary metal ion of the enzymes that catalyzed the biosynthesis of nucleosides and bases in *C. militaris*.

The biomass of cultured *C. militaris* reached a maximum at the 4th or 5th day, whereas maximum nucleosides and bases accumulation was obtained after 6 days' cultivation. The time profile of metabolites production was not consistent with that of growth, this was often the case in fermentation kinetics of higher fungi (Kim et al., 2002). Since the biomass of *C. militaris* at 6th day did not decrease much, it was feasible to prolong the time of harvest from the 5th to 6th day.

In conclusion, the best fermentation conditions for accumulation of 3′-deoxyadenosine, adenosine, guanosine, cytidine, uridine, adenine and uracil were set as follows: 20% potato broth, 2% glucose, 0.3% yeast extract, 0.3% peptone, 0.1 mmol/l Mn^{2+}, 0.05% $MgCl_2$ and 0.1% KH_2PO_4, and the duration of fermentation was 6 days. The information obtained in this work was helpful and useful to the development of *C. militaris* cultivation process for efficient production of these nucleosides and bases.

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