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Effects of polyamine biosynthesis inhibitors on *S*-adenosylmethionine synthetase and *S*-adenosylmethionine decarboxylase activities in carrot cell cultures

Subhash C. Minocha, Rakesh Minocha and Atsushi Komamine

Biological Institute, Faculty of Science, Tohoku University, Aramaki-Aoba, Sendai, Japan.

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Changes in the activites of *S*-adenosylmethionine (SAM) synthetase (methionine adenosyltransferase, EC 2.5.1.6.) and SAM decarboxylase (EC 4.1.1.50) were studied in carrot (*Daucus carota*) cell cultures in response to 2,4-dichlorophenoxyacetic acid (2,4-D) and several inhibitors of polyamine biosynthesis. Activity of SAM synthetase increased significantly during the first 5 days of culture under all conditions, maximum activity being observed in the presence of 2,4-D. DL-α-Difluoromethylarginine (DFMA), DL-α-difluoromethylornithine (DFMO) and cyclohexylammonium sulfate (CHAS) had little effect on the activity of this enzyme. The enzyme activity was lower than the controls in the presence of methylglyoxal bis(guanylhydrazone) (MGBG). The activity of SAM decarboxylase, which showed a peak on day 2 in all treatments, was lowered by 2,4-D, DFMA, MGBG and CHAS. The results are consistent with published reports on the effects of these inhibitors on the cellular levels of polyamines.

Additional key words – Cyclohexylammonium sulfate, 2,4-dichlorophenoxyacetic acid, difluoromethylarginine, difluoromethylornithine, methylglyoxal bis(guanylhydrazone).

S. C. Minocha (reprint requests), Department of Plant Biology, University of New Hampshire, Durham, NH 03824, *U.S.A.; R. Minocha, Louis C. Wyman Forest Sciences Laboratory, Northeastern Forest Experiment Station, Durham, NH* 03824, *U.S.A.; A. Komamine, Biological Institute, Faculty of Science, Tohoku University, Aramaki-Aoba, Sendai* 980, *Japan.*

Résumé. *L'évolution des activités* S-*adénosylméthionine* (*SAM*) *synthétase* (*méthionine adénosyltransférase, EC* 2.5.1.6) *et SAM décarboxylase* (*EC* 4.1.1.50) *a été étudiée sur des cultures de cellules de Carotte* (Daucus carota) *en réponse à l'activité de l'acide* 2,4-*dichlorophénoxyacétique* (2,4-*D*) *et de plusieurs inhibiteurs de la synthèse des polyamines. L'activité de la SAM synthétase augmente sensiblement durant les cinq premiers jours de la culture, dans toutes les conditions, l'activité maximale étant observée en présence de* 2,4-*D. La* DL-α-*difluorométhylarginine* (*DFMA*), *la* DL-α-*difluorométhylornithine* (*DFMO*) *et le sulfate de cyclohexylammonium* (*CHAS*) *n'ont que peu d'effet sur l'activité de cette enzyme. L'activité de l'enzyme est plus faible que dans les témoins en présence de méthylglyoxal bis*(*guanylhydrazone*) (*MGBG*). *L'activité de la SAM décarboxylase, qui présente un pic le deuxième jour dans tous les traitements, est abaissée en présence de* 2,4-*D, DFMA, MGBG et CHAS. Ces résultats sont en accord avec les résultats publiés sur les effets de ces inhibiteurs sur les teneurs des cellules en polyamines.* Mots clés additionnels : sulfate de cyclohexylammonium, acide 2,4-dichlorophénoxyacétique, difluorométhylarginine, difluorométhylornithine, méthylglyoxal bis(guanylhydrazone).

Abbreviations. ACC, 1-aminocyclopropane-1-carboxylic acid; ADC, arginine decarboxylase; CHAS, cyclohexylammonium sulfate; DFMA, DL-α-difluoromethylarginine; DFMO, DL-α-difluoromethylornithine; MGBG, methylglyoxyl bis(guanylhydrazone); ODC, ornithine decarboxylase; SAM, *S*-adenosylmethionine; dcSAM, decarboxylated *S*-adenosylmethionine; SAMDC, *S*-adenosylmethionine decarboxylase.

INTRODUCTION

An increased biosynthesis of polyamines (putrescine, spermidine, spermine) is a prerequisite for the development of somatic embryos in carrot cell cultures (Montague *et al.*, 1978, 1979; Feirer *et al.*, 1984; Fienberg *et al.*, 1984; Robie and Minocha, 1989; Mengoli *et al.*, 1989). This development is generally accompanied by increased activity of ADC, ODC and SAMDC. Several inhibitors of these enzymes have been employed to block the biosynthesis of polyamines in order to understand their role in embryogenesis and morphogenesis (Slocum *et al.*, 1984; Fobert and Webb, 1988; Minocha, 1988; Burtin *et al.*, 1989).

S-Adenosylmethionine is a key metabolite in polyamine biosynthesis. In addition to donating the aminopropyl moiety for spermidine and spermine biosynthesis, SAM also acts as a precursor for the biosynthesis of ethylene and as donor of methyl groups in a number of transmethylation reactions (Slocum *et al.*, 1984; Pegg, 1986; Miyazaki and Yang, 1987; Pegg and McCann, 1988). It has been suggested that in plants (*a*) the biosynthesis of polyamines competes with the biosynthesis of ethylene for this intermediate (Even-Chen *et al.*, 1982; Roberts *et al.*, 1984; Slocum *et al.*, 1984; Evans and Malmberg, 1989), and (*b*) this competition may be important in the control of senescence in some tissues and in the suppression of somatic embryogenesis in carrot (Roberts *et al.*, 1984; Minocha, 1988; Robie and Minocha, 1989). Because of its utilization in several pathways, it is conceivable that the biosynthesis of SAM is regulated independently from that of either ethylene or polyamines.

In animals and bacteria, SAM is synthesized largely by the enzyme SAM synthetase (methionine adenosyltransferase). The entry of SAM into the polyamine biosynthetic pathway is regulated by SAMDC (Slocum *et al.*, 1984; Pegg, 1986; Pegg and McCann, 1988; Smith, 1990). The product of this enzyme reaction (dcSAM) is generally not stored in the cells in any significant amounts, and it can be used only in the biosynthesis of spermidine and spermine (Greenberg and Cohen, 1985; Pegg, 1986; Kramer *et al.*, 1987, 1988; Pegg and McCann, 1988). It has also been reported that a depletion of the cellular levels of SAM causes a preferential reduction in the methylation of nucleic acids as compared to a decrease in polyamine biosynthesis (Kramer *et al.*, 1987, 1988). Whereas the existence of SAMDC and the modulation of its activity by polyamine biosynthetic inhibitors have been studied in some plants (Malmberg and Rose, 1987; Miyazaki and Yang, 1987; Malmberg and Hiatt, 1989), much less is known about SAM synthetase in plants.

This report is a part of studies in our laboratories on the role of polyamine and ethylene biosynthesis in somatic embryogenesis in carrot cell cultures. The effects of various inhibitors of polyamine biosynthesis on the activities of SAM synthetase and SAMDC are presented. Their effects on somatic embryogenesis and cellular polyamine levels have been reported elsewhere (Robie and Minocha, 1989; Minocha *et al.*, 1990; Khan and Minocha, 1990). Whereas SAMDC activity reflects changes in the cellular polyamine levels in response to inhibitors of polyamine biosynthesis, SAM synthetase activity is not affected by most of these inhibitors.

MATERIALS AND METHODS

Culture conditions. Cell cultures of carrot (*Daucus carota* L.) were acquired originally from Dr. D. F. Wetherell, University of Connecticut, in 1983. All stock cultures were maintained by a weekly subculture involving 1 : 10 dilution in 200 ml of MS medium (Murashige and Skoog, 1962) containing 2.26 μM 2,4-D. Routinely, the cultures are kept on a gyratory shaker (160 rpm) under 80 ± 10 μmol m^{-1} s^{-1} light at 25 ± 2°C; however, for the duration of the present studies, cultures were kept on a reciprocating shaker at 120 strokes per min in the dark. The medium was adjusted to a pH of 5.6 before autoclaving. Whereas 2,4-D was added before autoclaving, all inhibitors were filter-sterilized and added to the autoclaved media cooled to room temperature. The concentrations of inhibitors used in these experiments (2,4-D, 2.26 μM; DFMO, 1.0 mM; DFMA, 0.1 mM; CHAS, 0.5 mM; MGBG, 0.1 mM) were

selected on the basis of their effects on somatic embryogenesis and polyamine biosynthesis (Robie and Minocha, 1989; Minocha *et al.*, 1990; Khan and Minocha, 1990). Before transfer to experimental media, the cell suspensions were passed through a 193 µm sieve. The callus clumps retained on the sieve were discarded. The clumps passing through the sieve were washed 4 times with auxin-free medium by centrifugation (100 g for 2 min) and distributed to the experimental media (approximately 200 mg fresh weight per 50 ml medium) using a wide-mouth, 10 ml pipet. For experiments reported here, the inhibitors were tested only in the auxin-free medium. Each experiment was repeated at least three times. At each time either two or three replicate tissue samples were homogenized separately and assayed for enzyme activity. Data were analyzed by One-way Analysis of Variance using STATA version 1.5 (Computing Resource Center, Los Angeles, CA).

Determination of S-adenosylmethionine synthetase. The activity of *S*-adenosylmethionine synthetase (methionine adenosyltransferase, EC 2.5.1.6) was determined by a modification of the procedure of Hoffman (1983) and Markham *et al.* (1983). The cells were collected by vacuum filtration on a 32 µm nylon sieve, rinsed with distilled water, weighed and homogenized in the extraction buffer (1 ml per 250 mg fresh weight) using a Polytron homogenizer (Kinematica GmbH, Littan). The extraction buffer contained 0.1 M HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid]) (pH 7.5), 100 mM KCl, 15 mM $MgCl_2$ and 4 mM dithiothreitol. The homogenates were centrifuged at 19,000 *g* for 20 min and the supernatant fractions used for enzyme assays. Enzyme activity was determined by measuring the formation of radioactive *S*-adenosylmethionine from L-[methyl-^{14}C] methionine and ATP (Markham *et al.*, 1983). The reaction mixture in a total volume of 200 µl contained: 20 µl of 100 µM ATP, 20 µl of 10 mM [^{14}C] methionine (sp. act. 10 kBq mmol^{-1}, New England Nuclear) and 160 µl of the supernatant fraction. Following incubation at 37°C for 60 min, 80 µl of the reaction mixture were spotted on Whatman P-81 phosphocellulose paper (25 mm dia, numbered with pencil). The papers from different assays (maximum 20 per batch) were dropped in a solution of 0.1 M ammonium formate (pH adjusted to 3.1 with formic acid; total volume at least 10 ml per filter disc) and stirred slowly for 30 min. The filter discs were washed twice with fresh ammonium formate, twice with distilled water, and twice with absolute ethanol. The filter discs were oven-dried at 55°C for 30 min and placed in scintillation vials for counting of radioactivity. Blank assay mixtures contained homogenization buffer instead of the tissue extract. The procedure for enzyme activity measurements was optimized with respect to pH and temperature, and the reaction rate was linear for at least 90 min. Enzyme specific activity is expressed as nmol SAM synthesized h^{-1} mg^{-1} protein.

Determination of SAM decarboxylase. *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) activity was assayed by modification of the procedure of Pegg and Pösö (1983). The cells (250 mg fresh weight) collected by filtration as described above, were homogenized in 1 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 3 mM putrescine and 1 mM dithiothreitol using a Polytron homogenizer for 90 seconds. The homogenates were centrifuged at 19,000 *g* for 20 min and the supernatant fractions used for enzyme assays. The enzyme activity was determined by measuring the amount of $^{14}CO_2$ released from *S*-[carboxyl-^{14}C]-adenosyl-L-methionine as previously described for ODC (Khan and Minocha, 1989; Robie and Minocha, 1989). Briefly, 200 µl of the homogenate were mixed with 50 µl of 1.2 mM *S*-adenosylmethionine containing a final concentration of 1 kBq of radioactive SAM (sp. act. 569 MBq mmol^{-1}, New England Nuclear). The reaction was run in 16 × 100 mm Kimble test tubes, each fitted with a rubber stopper holding a polyethylene well (Kontes Scientific Instruments, Vineland, N.J.). A 2 cm^2 piece of Whatman 3 MM filter paper soaked with 50 µl Tissue Solubilizer (NCS, New England Nuclear) was placed in the well to trap $^{14}CO_2$. Tubes were incubated at 37°C for 30 min, following which 200 µl of 0.5 N H_2SO_4 were injected through the stopper. The tubes were incubated for an additional 30 min. The filter papers were removed and counted for radioactivity. Blanks contained the extraction buffer instead of the supernatant. The procedure was optimized with respect to pH and temperature and the reaction rate was linear up to 45 min. Specific activity of the enzyme is expressed as nmol CO_2 released h^{-1} mg^{-1} protein. The concentration of protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

S-Adenosylmethionine synthetase

Cultures grown in an auxin-free medium differentiated into somatic embryos within 7-12 days, but somatic embryogenesis was not detected in the presence of 2,4-D (see also Robie and Minocha, 1989). A time course of changes in the activity of SAM synthetase in carrot cells grown in the absence (control) and the presence of 2,4-D is

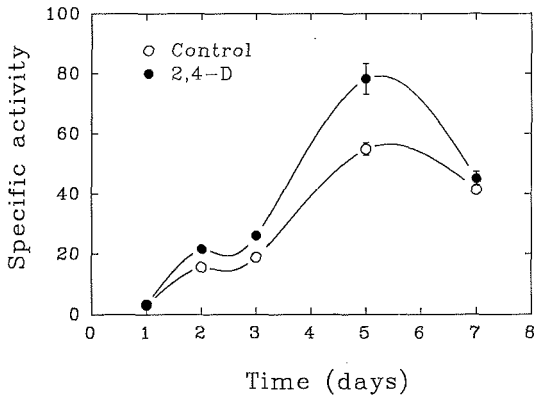

Figure 1. *Time course of changes in the activity of SAM synthetase in carrot cell cultures grown in the absence or in the presence of* 2.26 μ*M* 2,4-*D*. Enzyme activity is expressed as nmol SAM synthesized h^{-1} mg^{-1} protein. Each point is a mean of two replicates. SE bars where not shown are smaller than the symbols.

shown in figure 1. Enzyme activity increased rapidly during the first 5 days of culture in both cases. By the end of 5 days, specific activity of the enzyme in the control cells was more than 25-fold higher than on the first day, but by day 7 the enzyme activity had fallen slightly. The activity of this enzyme was always higher in cultures grown in the presence of 2,4-D as compared to those grown in the auxin-free medium. Whereas CHAS had no effect on the activity of the enzyme (*fig.* 2),

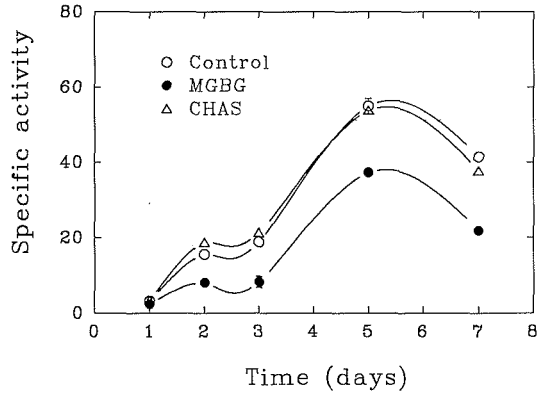

Figure 2. *Time course of changes in the activity of SAM synthetase in carrot cell cultures grown in the absence of* 2,4-*D and in the presence of either* 0.1 *mM MGBG or* 0.5 *mM CHAS.* Enzyme activity is expressed as nmol SAM synthesized h^{-1} mg^{-1} protein. Each point is a mean of two replicates. SE bars where not shown are smaller than the symbols.

MGBG caused a significant reduction by day 2 of treatment. Beyond this point, the activity of SAM synthetase was always lower in the MGBG-treated cells than in the control cells. However, there was a consistent increase in enzyme activity during the first 5 days of culture in all treatments. Neither DFMO nor DFMA had a significant effect on SAM synthetase activity during this period (data not shown).

S-Adenosylmethionine is involved in a number of metabolic reactions in the cell including the biosynthesis of polyamines and ACC and the methylation of nucleic acids (Slocum *et al.*, 1984; Pegg, 1986; Kramer *et al.*, 1987, 1988; Miyazaki and Yang, 1987; Pegg and McCann, 1988). It seems likely, therefore, that mechanisms for ensuring the availability of SAM would remain functional even if one or two of these pathways were significantly inhibited. This may explain why SAM synthetase activity increases during the culture period and why inhibitors of polyamine biosynthesis have little effect on this enzyme.

We have reported elsewhere (Minocha *et al.*, 1990) that MGBG, which is a potent inhibitor of SAMDC (Slocum *et al.*, 1984; Pegg, 1986), markedly lowers the cellular levels of spermidine and spermine in carrot cultures. Concomitant changes in the activity of SAM synthetase and cellular levels of spermidine and spermine in response to MGBG indicate that the biosynthesis of SAM may, at least to some extent, be related to its utilization rates in the polyamine biosynthetic pathway. This is especially relevant since decarboxylated SAM is not stored in the cells (Slocum *et al.*, 1984; Greenberg and Cohen, 1985; Pegg, 1986). This is, however, contradicted by the presence of a higher level of SAM synthetase in the presence of 2,4-D, when the cellular polyamine levels are actually lower (Feirer *et al.*, 1984; Robie and Minocha, 1989). Similarly, the lack of any significant effect of either DFMO (which promotes cellular polyamine levels) or DFMA (which inhibits cellular polyamine levels) (Robie and Minocha, 1989) also argues against a modulation of cellular SAM synthetase in relation to polyamine synthesis.

A common response of these cells to both MGBG and 2,4-D is an increased production of ACC (Minocha *et al.*, 1990; Samuelsen, 1990), which is also derived directly from SAM (Yang and Hoffman, 1984). Therefore, the SAM synthetase activity may be related more to the biosynthesis of ACC and ethylene than to the biosynthesis of polyamines. Thus the mechanism of regulation of SAM synthetase must await a detailed analysis of

the actual pool sizes and turnover rates of SAM, and its utilization in the biosynthesis of polyamines and ACC and the various methylation reactions. Such an analysis is currently under way in our laboratory at UNH.

Previous studies with animal tissues have shown that the inhibition of SAM biosynthesis causes a preferential decrease in methylation of nucleic acids and only a small decrease in polyamine biosynthesis (Kramer *et al.*, 1987, 1988). It has been further suggested that the inhibition of SAM synthesis results in compensatory increases in the activities of ODC and SAMDC. No corresponding work on the effect of polyamine biosynthetic inhibitors on SAM synthetase has been reported with plants to make such comparisons.

S-adenosylmethionine decarboxylase

S-adenosylmethionine decarboxylase is a key regulatory enzyme in the biosynthesis of spermidine and spermine (Slocum *et al.*, 1984; Pegg, 1986; Pegg and McCann, 1988; Smith, 1990). The product of its reaction (*i.e.* dcSAM) is the donor of aminopropyl moieties for these polyamines. Generally, decarboxylated SAM pools are very low in the cells, indicating that the activity of SAMDC is tightly regulated in relation to the biosynthesis of spermidine and spermine (Greenberg and Cohen, 1985).

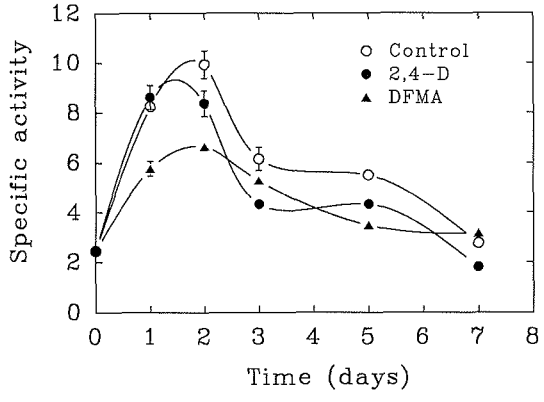

Figure 3. *Time course of changes in the activity of SAM decarboxylase in carrot cell cultures grown in the absence or in the presence of either* 2.26 µ*M* 2,4-*D or* 0.1 *mM DFMA.* Enzyme activity is expressed as nmol CO_2 released h^{-1} mg^{-1} protein. Each point is a mean of three replicates. SE bars where not shown are smaller than the symbols.

Data presented in figure 3 show that irrespective of the presence or the absence of auxin, the activity of SAMDC increased more than three-fold within the first day. A marked decline in enzyme activity was observed beyond day 2 such that by day 7 the activity was comparable to that in the starting cultures. A similar rapid increase in SAMDC both in embryogenic and non-embryogenic cultures of carrot was also reported by Montague *et al.* (1979). This increase was attributed to a "fresh medium effect" (Montague *et al.*, 1979). The increased activity during the first two days preceded the increased biosynthesis of spermidine and spermine seen in this tissue (Robie and Minocha, 1989; Khan and Minocha, 1990). Enzyme activity was significantly higher in cultures grown in auxin-free medium than in the auxin-supplemented medium. In contrast, Montague *et al.* (1979) did not observe any dfferences in SAMDC activity between the embryogenic and the non-embryogenic cultures. However, their studies on SAMDC were limited to the first 48 h of culture after transfer to the fresh medium. It has been repeatedly shown that differentiating carrot cultures (grown in auxin-free medium) possess higher titers of spermidine and spermine compared to non-differentiating tissues (grown in 2,4-D-supplemented medium) (Feirer *et al.*, 1984; Robie and Minocha, 1989).

In the presence of DFMA which inhibits polyamine biosynthesis as well as somatic embryogenesis in carrot cells (Feirer *et al.*, 1984; Robie and Minocha, 1989), a reduction in SAMDC was seen during the first 5 days (*fig.* 3). Likewise, in the presence of MGBG, another strong inhibitor of both spermidine and spermine biosynthesis and somatic embryogenesis, the activity of this enzyme

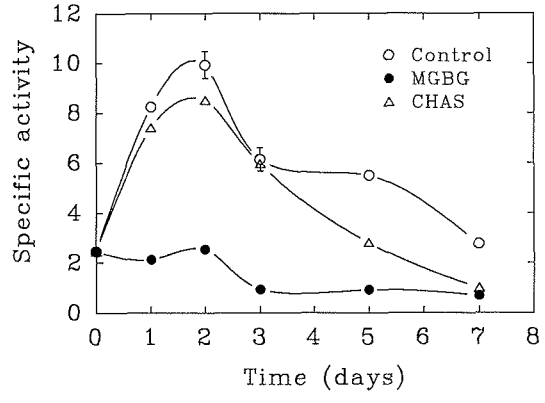

Figure 4. *Time course of changes in the activity of SAM decarboxylase in carrot cell cultures grown in the absence of* 2,4-*D and in the presence of either* 0.1 *mM MGBG or* 0.5 *mM CHAS.* Enzyme activity is expressed as nmol CO_2 released h^{-1} mg^{-1} protein. Each point is a mean of three replicates. SE bars where not shown are smaller than the symbols.

was significantly lower. The effect of MGBG was detected within 1 day of treatment and persisted throughout the 7 days of growth (*fig.* 4). Cyclohexylammonium sulfate was less effective than MGBG in lowering the enzyme activity and DFMO had no effect (data not presented).

Whereas the changes in SAMDC in response to 2,4-D, DFMA and CHAS are consistent with their effects on cellular polyamine levels (Robie and Minocha, 1989; Minocha *et al.*, 1990), the results with MGBG are at variance with published reports on the behavior of cellular SAMDC activity in response to this drug. MGBG which inhibits the activity of SAMDC *in vitro* is also known to stabilize the enzyme, resulting in an apparent increase in enzyme activity in the tissue in long term treatments (Pegg, 1986; Malmberg and Rose, 1987; Evans and Malmberg, 1988). No such increase in SAMDC activity was seen in carrot cells grown in the presence of MGBG for up to 7 days. There are no published reports on the effects of DFMO, DFMA and CHAS on SAMDC activity in plants.

CONCLUSION

Taken together, these results indicate that due to a multipurpose metabolic role of SAM in the cell and its major use in methylation reactions, SAM synthetase activity remains quite high even when polyamine biosynthesis is severely inhibited. On the other hand, cellular SAMDC activity shows a strong correlation with the biosynthesis of polyamines.

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