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Malate Metabolism and Adaptation to Chilling Temperature Storage by Pretreatment with High CO_2 Levels in *Annona cherimola* Fruit

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In this study we focused on the effect of a pretreatment with high (20%) CO_2 levels on malic acid metabolism in cherimoya (*Annona cherimola* Mill) fruit stored at chilling temperature. We analyzed the activity of phospho*enol*pyruvate carboxylase (PEPC), malate dehydrogenase (MDH), and the NADP−malic enzyme (NADP−ME), involved in the carboxylation/decarboxylation of malate. Our results show that CO_2 treatment, which improves tolerance to prolonged storage at chilling temperature, was closely linked to considerably greater NADP−ME activity. These results, combined with lower PEPC activity, may explain the significantly lower amount of malic acid and titratable acidity quantified in CO_2-treated fruit. Moreover, the high cytoplasmic MDH enzyme activity and the strong stimulation of NADP−ME activity exhibited by CO_2-treated fruit could be contributing factors in the maintenance of fruit energy metabolism, pH stability, and the promotion of synthesis of defense compounds that prevent or repair damage caused by chilling temperature.

KEYWORDS: Cherimoya; chilling temperature; titratable acidity; malate; carbon dioxide; phosphoenolpyruvate carboxylase; malate dehydrogenase; malic enzyme

INTRODUCTION

The responses of fruits to high CO_2 levels vary considerably among cultivars and depend on environmental conditions of storage (*1*, *2*). Controlled atmospheres, with high CO_2 and low O_2 concentrations, have been shown to control some physiological disorders, thereby extending the storage period of fruit and vegetables (*3*). Cherimoya fruit is a chilling-sensitive fruit in which the occurrence of chilling injury (CI) is readily assessed by changes in pH evolution and accumulation of citrate constitute (*4*, *5*). Reports on the application of controlled atmospheres with high CO_2 levels suggest that these help prevent CI in cherimoya fruit by modulating the phenolic response associated with cell adhesion and hardening (*6*), maintaining flesh firmness and chlorophyll content, and maintaining levels of ribulose 1,5-biphosphate carboxylase and polygalacturonase-related proteins (*7*). Some of the beneficial effects of high CO_2 levels in the prevention of stress conditions are due to the accumulation of γ-aminobutyric acid together with spermidine and spermine; it has been suggested that they may be an early adaptive response of plant tissues to different stresses (*8*−*11*). Moreover, some of these compounds are related to the activation of reactions to maintain the pH-stat of plant cells. It has been suggested that the capacity to regulate pH may be a key factor in determining species survival under stressful conditions (*12*). Malate metabolism regulation plays an important role in cytoplasmic pH control (*13*−*15*). Malate is known to be involved in a wide variety of physiological processes, and its accumulation is subject to considerable changes under different physical, chemical, and pathogen stresses (*16*, *17*). Likewise, fluctuations in the levels of malate and changes in the activities of malate-transforming enzymes upon different stress conditions suggest that malate metabolism has a role in plant defense (*16*, *18*). On the other hand, there has been no research into the role of high CO_2 levels in reducing the effects of abiotic stresses in fruits and vegetables through the regulation of malate metabolism.

For the regulation of malate metabolism, the rate at which malate is produced via the cytosolic enzymes phospho*enol*pyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) must be closely matched by the rate of malate decarboxylation by cytosolic NADP−malic enzyme (NADP−ME). It has been suggested that PEPC and MDH also function as a pH-stat, controlling cytoplasmic pH by balancing malate synthesis and degradation (*19*−*21*). PEPC (EC 4.1.1.31) is a ubiquitous cytosolic enzyme that catalyzes the irreversible β-carboxylation of phosphoenolpyruvate (PEP) by HCO_3^- in the presence of divalent cation to yield Pi and oxaloacetate (OAA). It is an important enzyme for the carbon economy of the cell, and several functions have been attributed to the PEPC enzyme in nonphotosynthetic tissues, such as NADPH generation, recapture of respired CO_2, malate fermentation, pH stability and main-

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tenance of electroneutrality, and replenishment of tricarboxylic acid cycle intermediates (*22*). This last function of PEPC, also known as the anaplerotic function, provides the carbon skeletons necessary for nitrogen assimilation (*23*). In cherimoya fruit, such a connection between carbon and nitrogen metabolisms has already been proposed (*24*) and its specific properties and regulation by a variety of metabolites have been described (*25*).

MDH (EC 1.1.1.37) converts the OAA produced by PEPC to malate. This reaction is important in cellular metabolism and is coupled with easily detectable cofactor oxidation/reduction. The diversity of roles of plant MDHs is reflected in their variety of subcellular localization and cofactor NAD or NADP specificities (*13*, *26*).

ME (EC 1.1.1.40) is a widely distributed enzyme that catalyzes the oxidative decarboxylation of malate. Cytosolic forms of NADP−ME seem to be present in all plants and fulfill diverse housekeeping functions due to their universal presence in different plant tissues. Some of the suggested roles include a contribution to the maintenance of intracellular pH and catabolic functions consisting in the channeling of NADPH and pyruvate into respiratory pathways for energy production (*27*).

The aim of this work was to determine whether variations in the titratable acidity and malate concentration resulting from high CO_2 levels to overcome chilling damage can be explained in terms of the biochemical and metabolic characterization of the main enzymes involved in the carboxylation/decarboxylation system.

MATERIALS AND METHODS

Plant Material. Cherimoya (*Annona cherimola* Mill. cv. 'Fino de Jete') fruits of uniform maturity and size and free from physical and pathological defects were harvested in Almuñecar (Granada, Spain) in October. Selected fruits were randomly divided into two lots of 60 fruits and stored in the dark at 6 °C in separate respiration chambers (20 L) under a continuous flow (100 mL/min) of humidified air (nontreated fruit) or a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit). After 3 days, CO_2-treated fruits were transferred to a continuous humidified airflow. CO_2-treated and nontreated fruits were sampled at the same chronological age after 3, 5, 9, 16, and 23 days of storage at 6 °C. For biochemical analysis, cherimoyas were sampled, peeled, frozen in liquid nitrogen, and stored at −80 °C until use.

Titratable Acidity. Titratable acidity was determined using a Mettler DL70 automatic titrator with a serially connected interface for the ST20 sample changer and a current loop interface for a Mettler AT100 balance that automatically transfers the sample weight. This parameter was determined twice per fruit. A 10 g amount of pulp was diluted and homogenized in 20 mL of Milli-Q deionized water (Milli-Q system, Millipore, Milford, MA). Titration was performed with 0.1 N NaOH up to pH 8.1 (AOAC standard). Total acidity refers to malic acid content since this is the predominant organic acid in cherimoyas. Results were expressed as percent of malic acid and converted to mequiv of malic acid per 100 g of fresh weight.

Organic Acid Determinations. A 10 g amount of pulp was homogenized in 100 mL of methanol with an Omnimixer (Waterbury, CT) at 7000 rpm for 5 min. The homogenate was refluxed at 50 °C for 15 min and then filtered under vacuum. The methanol was evaporated under vacuum in a rotary evaporator at 40 °C, and the residue was resuspended in 50 mL of Milli Q water and passed through a methanol activated Sep-Pack C_{18} minicolumn (Waters, Milford, MA). The eluate was filtered through a 0.45 μm Millipore filter, and 20 μL was injected in the high-performance liquid chromatography equipment (Waters). Organic acids were separated on a 30 cm × 6.5 mm ION-300 column (Interaction Chemicals, Mountain View, CA) at 45 °C using 0.01 N H_2SO_4 as a solvent (flow rate of 0.4 mL/min) and detected by ultraviolet absorption at 214 nm (Waters detector, model 441). Quantitative assessment was based on external standards and expressed as milligrams of malate or citrate acid per gram of fresh weight.

PEPC, MDH, and NADP−ME Extraction and Activities. Protein extracts for the PEPC assay were obtained by homogenizing ground, frozen mesocarp cherimoya tissue (2.5 g) at 4 °C in 7.5 mL of 50 mM Tris-HCl (pH 7.8) containing 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 2 mM DTT, 0.25 mM EDTA, and 2% (w/v) PVPP. The homogenate was centrifuged at 20 000 *g* for 30 min at 4 °C. PEPC activity was determined spectrophotometrically at 340 nm (Perkin-Elmer, Inc., Lambda 15 UV/VIS) by coupling the reaction to NADH oxidation in the presence of MDH. The standard assay medium contained 0.2 mL of crude enzyme extract (which contained endogenous MDH activity), 0.14 mM NADH, 10 mM $MgCl_2$, 12 mM $NaHCO_3$, 10 mM DTT, 0.25 mM EDTA, and 50 mM Tris-HCl buffer (pH 7.8). The reaction was started by addition of PEP (final concentration 4 mM) to the spectrophotometer cuvette containing the assay mixture at 27 °C. The rate of oxidation of NADH was measured in the linear range (1 min). Enzyme activity was expressed as micromoles of PEP consumed per minute per milligram of protein.

Protein extracts for MDH and NADP−ME assays were obtained by homogenizing ground frozen mesocarp cherimoya tissue (2.5 g) at 4 °C in 10 mL of 0.1 M Bicine−0.1 M MOPS (pH 7.0) containing 0.3 mM DTT, 4 mM EDTA, 5% (v/v) PEG, and 1% (w/v) PVP. MDH activity was assayed by monitoring oxidation of the reduced form of NADH measured at 340 nm in a reaction mixture containing 0.1 M MOPS buffer (pH 7.0), 3.33 mM OAA, and 0.2 mM NADH at 27 °C. Enzyme activity was expressed as millimoles of NADH consumed per minute per milligram of protein.

NADP−ME activity was assayed by monitoring production of the reduced form of NADPH measured at 340 nm in a reaction mixture containing 0.1 M MOPS buffer (pH 7.0), 17 mM sodium malate, 4.5 mM $MnSO_4$, and 0.7 mM $NADP^+$ at 25 °C. Enzyme activity was expressed as millimoles of $NADP^+$ consumed per minute per milligram of protein.

Protein Estimation. Protein concentration was measured by the method of Bradford (*28*) using a protein−dye reagent (Bio-Rad) and BSA as a standard.

Statistical Analyses. Data from at least three replicates per sample were subjected to analysis of variance (ANOVA) at $P = 0.05$ (Statgraphics program, STSC, Rockville, MD). The main effects of CO_2 treatment, time of storage at 6 °C, and treatment × time interaction on fruit were analyzed (*F* values). Correlations (Spearman Rank) were established among the different parameters studied to assess the effect of CO_2 treatment on malate metabolism. Correlation coefficients (*r* values) were estimated as acceptable about 0.50 and as good higher than 0.70. The results presented here represent data from three or more experiments performed in succession.

RESULTS

Titratable Acidity and Levels of Citric and Malic Acids in CO_2-Treated and Nontreated Fruit During Storage at Chilling Temperature. Titratable acidity increased considerably (by around 75%) between days 6 and 16 in the nontreated fruits; in the CO_2-treated fruits the increase was smaller and occurred later (Figure 1A). In the nontreated fruits, the malate content peaked on day 16, coincident with maximum titratable acidity, while in the CO_2-treated fruits the malic acid content was lower but reached the maximum at the same time (Figure 1B). At the end of storage, there was a decrease in both titratable acidity and malate content in nontreated fruits (Figure 1A,B). However, in the CO_2-treated fruits there was no change in either the titratable acidity or the malate content.

The concentration of citric acid in freshly harvested fruits was 2 times lower than the malate content. In nontreated fruit, the level increased progressively during low-temperature storage, so that after 23 days the citric acid levels reached 1.33 ± 0.09 mg/g FW, which is more than twice the levels found in freshly harvested fruits. However, the maximum citric acid content reached after 23 days was 3 times lower than malate levels. Although the citric acid content in nontreated and CO_2-treated

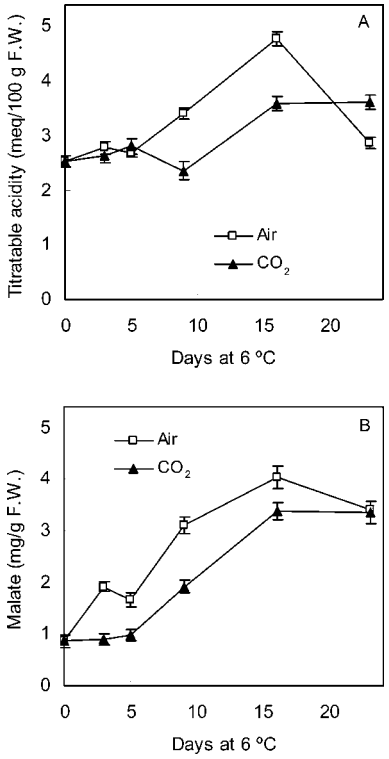

Figure 1. Titratable acidity (A) and malate content (B) in nontreated and 20% CO_2-treated cherimoya fruit stored at 6 °C. Vertical bars indicate ±SE.

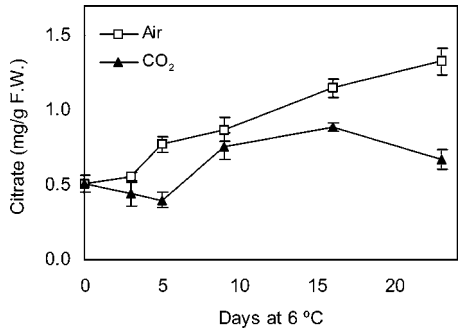

Figure 2. Changes in citrate levels in nontreated and 20% CO_2-treated cherimoya fruit stored at 6 °C. Vertical bars indicate ±SE.

fruits was similar at the beginning of the storage period, citric acid levels in treated fruits were significantly lower over prolonged storage ($P = 0.05$) (Figure 2).

Carboxylation/Decarboxylation System in CO_2-Treated and Nontreated Fruit During Storage at Chilling Temperature. In nontreated fruit, PEPC activity increased sharply after 3 days of storage at chilling temperature; thereafter the activity decreased progressively reaching similar values to those achieved in freshly harvested fruits (Figure 3A). At the end of storage, the activity increased drastically, and after 23 days the values were 5 times higher than in freshly harvested fruits. In CO_2-treated fruit, the low initial PEPC activity levels increased after 16 days, although maximum values were one-half those of nontreated fruits.

The activity of the cytoplasmic MDH declined significantly ($P = 0.05$) in treated and nontreated fruits up to 5 days of storage at 6 °C (Figure 3B). However, in nontreated fruits the MDH activity recovered progressively up to day 16 and declined after 23 days. On the contrary, in the CO_2-treated fruits the MDH activity increased continuously from day 5 until the end of storage.

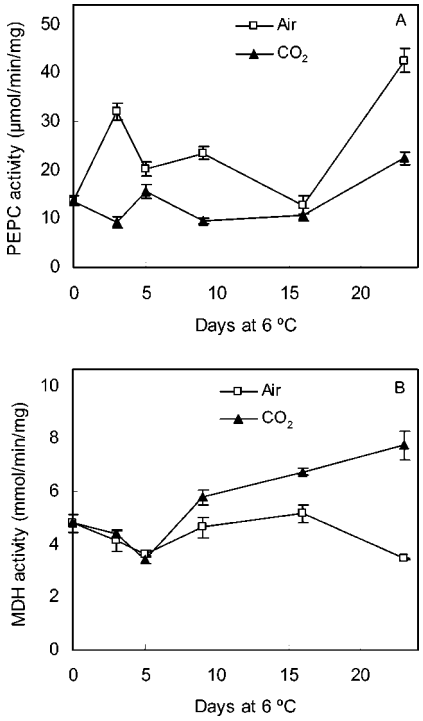

Figure 3. PEPC (A) and MDH (B) activities in nontreated and 20% CO_2-treated cherimoya fruit stored at 6° C. Vertical bars indicate ±SE.

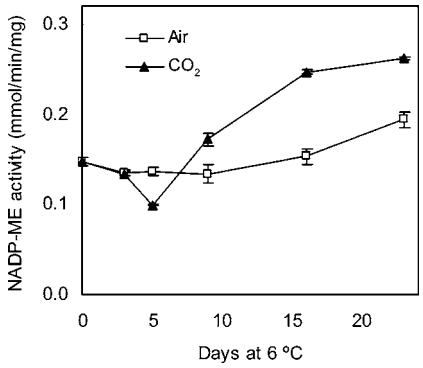

Figure 4. NADP–ME activity in nontreated and 20% CO_2-treated cherimoya fruit stored at 6 °C. Vertical bars indicate ±SE.

Table 1. *F* Value and Significant Level[a] for the Effects of 20% CO_2 Treatment and Time on Malate Metabolism and Titratable Acidity in Cherimoya Fruit Stored at 6 °C

factor	PEPC	MDH	ME	malate	titratable acidity
CO_2	467.9[a]	136.8[a]	195.3[a]	144.1[a]	17.5[a]
days (D)	151.4[a]	52.7[a]	315.5[a]	371.1[a]	65.7[a]
$CO_2 \times D$	65.5[a]	46.1[a]	106.3[a]	15.9[a]	24.7[a]

[a] Significance at the 0.05 level.

As Figure 4 shows, NADP−ME activity was practically constant in nontreated fruit throughout storage. Nevertheless, in the CO_2-treated fruit, despite a slight decrease observed at day 5, NADP−ME activity increased progressively thereafter until the end of storage. In CO_2-treated fruit, the NADP−ME activity reached higher levels (up to 30%) than in nontreated fruit.

Statistical Analyses. ANOVA confirmed that the factors CO_2, time, and CO_2−time interaction significantly ($P = 0.05$) affect all the parameters studied (Table 1). Regarding the effect of the different factors analyzed, CO_2-treated fruits presented

Table 2. *r* of Correlations between Malate Metabolism Parameters in Nontreated and 20% CO_2-Treated Cherimoya Fruit Stored at 6 °C

	treatment	MDH	ME	malate
PEPC	nontreated	–0.63	0.08	0.23
	CO_2-treated	0.22	0.23	0.25
MDH	nontreated		0.06	0.15
	CO_2-treated		0.99	0.85
ME	nontreated			0.49
	CO_2-treated			0.83

higher *F* values for the variable PEPC activity than for NADP−ME, MDH, malate, and titratable acidity. With respect to the factor time, the *F* values were lowest for MDH and titratable acidity. However, statistical analysis confirmed that the factor CO_2−time interaction was the main influence on NADP−ME activity (higher *F* value) with respect to the others variables studied.

Correlation analysis showed that *r* values between malate content and titratable acidity were high, at 0.88 and 0.75 for nontreated and CO_2-treated fruit, respectively. Table 2 shows the correlation analysis for malate metabolism-related parameters. In general, in nontreated fruit *r* values between PEPC and MDH were low with the other parameters studied; only NADP−ME activity presented an acceptable correlation with malate content. On the other hand, CO_2-treated fruit presented good *r* values for MDH and NADP−ME with malate; the best correlations were between MDH and ME activities (Table 2).

DISCUSSION

Malate is a highly versatile metabolite which has several important functions in plants, including the maintenance of cytoplasmic pH, charge balance, and serving as a key metabolic intermediate (*13*). Moreover, there is evidence for the importance of malate and other organic acids in the response of plants to several environmental stresses (*29*, *30*). Our results showed a sustained upward trend of malate content, paralleling a sharp increase in titratable acidity, during the storage of nontreated cherimoyas at chilling temperature (Figure 1). The statistical analysis showed high *F* values of the factor time for the variables NADP−ME and malate but lower values for MDH (Table 1). The effect of time on MDH activity is related to intense MDH activity in freshly harvest fruit throughout low-temperature storage, which was much higher than in most fruit tissues (*31*, *32*). In nontreated fruit, depending on the malate content and the levels of malate-related enzyme activities, the rate of carboxylation vs decarboxylation of malic acid was high during storage at chilling temperatures. Moreover, in terms of enthalpy ($\Delta G = -1.5$ kJ mol^{-1}), low temperatures would favor accumulation as opposed to degradation of malic acid (*17*). In contrast, Roe et al. (*31*), examining the effect of low temperatures on the enzymes implicated in acid metabolism in orange, reported a pronounced reduction of PEPC, MDH, and ME activity. In nontreated cherimoyas, the increase in PEPC activity during storage at chilling temperature could be connected with the response to cold stress. In this connection, cold treatments have been reported to induce the progressive accumulation of PEPC transcripts in roots and shoots of wheat seedlings (*33*). Also, the induction of PEPC expression by salt stress has been described in *Mesembryanthemum crystallinum* (*34*, *35*). It has also been reported that nitrate induces the accumulation of malate and the expression of PEPC in tobacco leaves and roots (*36*).

ANOVA showed high *F* values for the effect of the CO_2 on PEPC activity, which could indicate that the pattern of activity for this enzyme is altered by gas treatment (Table 1). CO_2-treated fruit showed reduced PEPC activity during low-temperature storage; these changes could be related to the reported beneficial effect of high CO_2 treatment in preventing CI in cherimoya (*6*−*9*). It is also possible that in CO_2-treated fruit PEP is preferentially channeled to substrate-level ATP production instead of to malate production. However, at the end of the storage period (23 days) there was an increase in PEPC activity, which would seem to indicate that the protective effect of the CO_2 was progressively lost when storage was prolonged. Despite this possible loss of the protective effect of CO_2, at the end of storage the level of citrate, a compound that we previously reported as a marker of chilling damage in cherimoya fruit (*5*), was significantly higher in nontreated than in CO_2-treated fruit (Figure 2).

In CO_2-treated fruit, the total amount of malate and the titratable acidity value were significantly lower than in nontreated chilled fruit. Also, exposure to 20% CO_2 has been reported to reduce the malate content in lettuce tissues (*37*). The statistical analysis showed a good correlation coefficient between both MDH/NADP−ME activities and malate content (Table 2). Although the special significance of the high activity of cytoplasmic MDH is unknown, the reduction of OAA consumes NADH and protons; the higher activity observed in CO_2-treated fruit could serve to reduce cytoplasmic acidosis and replenish NAD^+, which is essential for the continuation of glycolysis, thus contributing to energy metabolism. Nevertheless, the factor CO_2−time interaction most affected NADP−ME activity, indicating that malic acid metabolism was mainly regulated by the effect of CO_2−time interaction on NADP−ME activity. The activation of NADP−ME by high CO_2 treatment could provide fruits with more reductive power and pyruvate that may be used for respiration in cellular repair processes and for synthesis of compounds that help prevent or repair damage caused by chilling temperature as previously reported (*6*, *8*, *9*). Specifically, we observed that CO_2 treatment altered the concentration and timing of accumulation of the phenolic compounds that seem to regulate the strength of cell adhesion and so prevent hardening caused by chilling temperature storage (*6*). In support of this, there have been reports of an increase in the activity of ME connected with a role of malate metabolism in plant defense (*38*) and adaptation of crassulacean acid metabolism plants to environmental condition (*39*).

Additionally, the intense stimulation of NADP−ME activity observed under high CO_2 treatment may be the key to resistance to one of the most damaging effects of chilling, namely, cytosolic acidification. We reported elsewhere that cytoplasmic acidification, as measured by nuclear magnetic resonance, is a fundamental feature of the early stages of cherimoya fruit storage at chilling temperatures (*25*). Activation of the decarboxylation reaction by CO_2 treatment could be a strategy for regulating the cellular pH or delaying acidification. It has been widely reported that decarboxylation acts as a sink for excess protons, delaying acidification, (*19*−*21*). Also, malate metabolism is quantitatively one of the main mechanisms used by acid-loaded cells of *Acer pseudoplatanus* to regulate their cytoplasmic pH (*40*). Given that the CO_2 treatment used to increase tolerance of chilling injury in cherimoya fruit triggers mechanisms that are able to counter the perturbations caused by chilling damage, our results tend to support the importance of NADP−ME enzyme for adaptation to chilling injury through regulation of malate levels.

To judge by the evolution of enzymes involved in the carboxylation/decarboxylation system as it relates to the concentration of malate, our results suggest that PEPC and NADP−ME activity could be used as a biochemical marker of fruit susceptibility/adaptation to stress conditions. Specifically, our results support the importance of NADP−ME enzyme in the adaptation of cherimoya fruit to prolonged storage at chilling temperature by means of high CO_2 levels.

ABREVIATIONS USED

CI, chilling injury; MDH, malate dehydrogenase; ME, malic enzyme; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase.

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