Intramolecular Carbon Isotopic Composition of Monosodium Glutamate: Biochemical Pathways and Product Source Identification

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Monosodium glutamate (MSG) obtained as trade samples from several manufacturers was studied to determine the range of its intramolecular $^{13}C/^{12}C$ composition. Although the carbon isotopic composition of the total MSG molecule did not differ among manufacturers in most instances, significant differences were observed in the isotopic composition of the α-carboxyl carbon, suggesting that different proprietary strains of industrial microorganisms or MSG purification methods may impart unique isotopic fingerprints upon their products. The ^{13}C depletion of the α-carboxyl carbon relative to the rest of the molecule helps constrain the identity of the potential anapleurotic carboxylating enzymes responsible for its fixation.

KEYWORDS: Monosodium glutamate; carbon isotopes; anapleurotic pathway; intramolecular isotope distribution

INTRODUCTION

It has long been recognized that the carbon skeletons of biological molecules can be isotopically heterogeneous. Indeed, these intramolecular isotopic differences appear to be the rule rather than the exception among biomolecules. The patterns of isotope distribution within molecules retain evidence of their origin, and the biochemical fluxes during their syntheses and intramolecular isotopic analyses have not been employed widely in the food industry; there are instances in which intramolecular isotopic information may be uniquely useful. Weber et al. (*1*, *2*) have examined the intramolecular isotopic composition of carbon atoms within glycerol, which is a minor fermentation product of wines. Glycerol attributes "body" to the wine, and there may be motivation by unscrupulous producers to adulterate wines by addition of small amounts of glycerol from outside sources. Weber et al. (*3*) found that authentic glycerols are greatly ^{13}C depleted at the C1 position. In contrast, glycerols from animal or abiogenic sources do not have the same depletion at the C1 position. Similarly, natural ascorbate retains its glucose precursor (*4*). Industrial synthesis of ascorbate yields ^{13}C enrichment at the C1 postion.

The application of intramolecular isotope data is not limited to product source identification. Because combinations of isotope effects and metabolic fluxes can yield a range of isotopic values for particular compounds, the magnitude of the isotope effect expressed at specific carbon positions within them can constrain the biochemical processes responsible for their synthesis. Such information is potentially useful for food scientists concerned with engineering organisms for optimal production or for process engineers interested in monitoring the status of industrial fermentations.

We were approached by an industrial partner to investigate whether it was possible to resolve sources of monosodium glutamate (MSG) using intramolecular carbon isotopic measurements. Because the MSG samples consisted of a purified product, they could not be resolved on the basis of unique molecular constituents. Because most of the samples were derived from isotopically similar sources (C4 plant sugars), they could not be distinguished consistently on the basis of their bulk carbon isotopic composition. However, the intramolecular distribution of isotopes and, specifically, the relative isotopic depletion of the α-carboxyl carbon of the MSG molecule, was distinct and could be used to distinguish among the products of different manufacturers. Differences in the intramolecular isotopic composition of the various MSG samples suggest that manufacturers may have adopted multiple solutions to the problem of engineering microbial metabolism for MSG production.

MATERIALS AND METHODS

Fifteen samples of MSG were obtained as trade samples from six manufacturers. The isotopic composition of the total MSG molecule was obtained by combustion in a Carlo Erba model 1108 CNS analyzer. CO_2 from the combustion was trapped cryogenically and transferred to a Pyrex breakseal tube (*5*). The isotopic composition of the CO_2 was measured on a Finnigan MAT Delta E isotope ratio mass spectrometer equipped with a modified inlet system for small samples (*6*). Combusted acetanilide standards averaged −0.04 ± 0.02‰ relative to the accepted values after blank correction. Blanks ($n = 27$) consisted of empty foil boats. The precision of triplicate analyses of MSG was ±0.01 ‰.

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Table 1. Sources and Isotopic Composition of MSG Samples (A) and Samples Pooled by the Manufacturer (B)[a]

(A) All MSG Samples

sample	$\delta^{13}C_{TOT}$		$\delta^{13}C_{COO}$		$\delta^{13}C_{RES}$		$\Delta\delta^{13}C_{TOT}$		$\Delta\delta^{13}C_{RES}$	
ADM	−11.55	0.01	−16.19	0.14	−10.39	0.03	−4.64	0.14	−5.81	0.17
TAKEDA "extra fine"	−14.18	0.01	−21.02	0.19	−12.44	0.05	−6.84	0.20	−8.55	0.24
TAKEDA "fine 941002"	−13.23	0.01	−19.71	0.19	−11.62	0.05	−6.48	0.20	−8.12	0.24
TAKEDA "fine 941102"	−13.28	0.01	−20.10	0.19	−11.54	0.05	−6.82	0.20	−8.56	0.24
TAKEDA "fine 941012"	−13.22	0.01	−19.06	0.19	−11.76	0.05	−5.84	0.20	−7.34	0.24
TAKEDA "fine"	−14.12	0.01	−21.16	0.19	−12.37	0.05	−7.04	0.20	−8.84	0.24
TAKEDA "regular"	−12.70	0.01	−20.86	0.19	−10.66	0.05	−8.16	0.20	−10.20	0.24
TAKEDA "standard"	−12.90	0.01	−20.75	0.27	−10.94	0.07	−7.85	0.28	−9.81	0.34
VEOAN "Taiwan"	−12.45	0.01	−16.02	0.27	−11.56	0.05	−3.57	0.28	−4.46	0.34
VEOAN "Tung Hai"	−12.46	0.01	−16.17	0.27	−11.53	0.05	−3.71	0.28	−4.64	0.34
AJINOMOTO "Iowa"	−13.14	0.01	−21.80	0.27	−10.98	0.07	−8.66	0.28	−10.83	0.34
AJINOMOTO "150692"	−12.42	0.01	−20.28	0.27	−10.46	0.07	−7.86	0.28	−9.83	0.34
AJINOMOTO "Accent"	−13.48	0.01	−23.62	0.27	−10.95	0.07	−10.14	0.28	−12.68	0.34
ASAHI	−13.93	0.01	−21.26	0.27	−12.10	0.07	−7.33	0.28	−9.16	0.34
ORSAN	−25.18	0.01	−32.08	0.14	−23.45	0.03	−6.90	0.14	−8.53	0.17

(B) Samples Pooled by the Manufacturer

manufacturer	$\delta^{13}C_{TOT}$		$\delta^{13}C_{COO}$		$\delta^{13}C_{RES}$		$\Delta\delta^{13}C_{TOT}$		$\Delta\delta^{13}C_{RES}$	
ADM	−11.55	0.53	−16.19	0.99	−10.39	0.56	−4.64	0.82	−5.81	1.03
ASAHI	−13.93	0.53	−21.26	1.02	−12.10	0.57	−7.33	0.86	−9.16	1.07
VEOAN	−12.46	0.37	−16.10	0.72	−11.55	0.40	−3.64	0.60	−4.55	0.76
AJINOMOTO	−13.01	0.31	−21.90	0.59	−10.80	0.33	−8.89	0.49	−11.11	0.62
TAKEDA	−13.38	0.20	−20.38	0.38	−11.63	0.21	−7.00	0.32	−8.75	0.39
ORSAN	−25.18	0.53	−32.08	0.99	−23.45	0.56	−6.90	0.82	−8.63	1.03

[a] Data are given as least-squares means ± one standard error.

The α-carboxyl carbon of the MSG molecule was isolated by chemical decarboxylation. Approximately 20 mM stock solutions of the MSG samples were made up in a pH 2.5 phosphate buffer. A 0.5 mL amount of the solution was pipetted into 3 mL glass serum vials (Wheaton) and frozen. To each vial was added 0.5 mL of a 0.4 M ninhydrin (2,2-dihydroxy-1,3-indandione; Pierce Chemical) solution. The vials were quickly capped with greased butyl rubber stoppers and crimp sealed. The decarboxylation reaction proceeds slowly at low temperatures (*7*), permitting the vials to be capped before any CO_2 was evolved from the reaction. The vials were dropped into a boiling water bath for 15 min to allow the reaction to proceed. Under the reaction conditions, ninhydrin quantitatively decarboxylated the α-carboxyl carbon of glutamate and released the carbon as CO_2. No contribution of the δ-carboxyl carbon could be detected under the reaction conditions.

After the incubation was complete, the vials were removed from the water bath and allowed to cool. A 0.1 mL amount of 1 M phosphoric acid and 0.1−0.2 mL of an antifoaming agent (Sigma Antifoam A) were injected into the vial through the rubber septum. A stream of helium was admitted into the solution through a needle piercing the vial cap. The helium stripped the CO_2 from solution. The CO_2 and helium carrier were passed through a second needle into a custom gas chromatograph (GC) equipped with a Nafion drying tube (PermaPure Products), a 1 m long × 2.2 mm i.d. steel column packed with Unibeads 1S (Alltech), and a thermal conductivity detector. The CO_2 was quantified, trapped cryogenically, and transferred to a Pyrex breakseal. Blanks (*n* = 14) consisted of sample-free buffer carried through the entire analytical procedure. Isotopic fidelity of the GC analysis was checked by analysis of standard bicarbonate solutions. The isotopic composition of standards averaged −19.46 ± 0.01‰. The accepted value for the standard was −19.5 ± 0.1‰. Precisions of replicated (*n* = 4) measurements of MSG from a single source were 0.4‰.

δ¹³C of samples were determined by comparison to secondary standards calibrated against the Pee Dee belemnite standard: $\delta^{13}C_{SAMPLE} = [(R_{SAMPLE}/R_{PDB}) − 1] \times 1000$, where R = the ratio of ¹³C/¹²C in the sample and the standard. The isotopic difference between the carboxyl carbon and the total MSG molecule was defined as $\Delta\delta^{13}C_{TOT} = \delta^{13}C_{COO} − \delta^{13}C_{TOT}$. The isotopic composition of the residual carbon ($\delta^{13}C_{RES}$)

of MSG could be calculated by difference. The isotopic difference between the carboxyl carbon and the residual carbon of a sample was defined as $\Delta\delta^{13}C_{RES} = \delta^{13}C_{COO} − \delta^{13}C_{RES}$.

Statistical comparisons of the isotopic composition of MSG among trade samples and among manufacturers were made using a mixed model analysis of variance (SAS PROC MIXED), with either manufacturer or sample analyzed as a fixed variable. Within the analysis, subsamples were nested as random factors within samples, and samples were nested as random factors within manufacturers. Degrees of freedom for the unbalanced data set were calculated using the Satterthwaite approximation (*8*). Data are reported as least-squares (population marginal) means ± one standard error, except where otherwise noted. Analysis of mixed models using SAS software is discussed in Littell et al. (*9*) and Verbeke and Molenberghs (*8*). Pairwise comparisons between samples or between manufacturers were made for instances in which the overall test was significant (*P* < 0.05) and were corrected for multiple testing using a Tukey−Kramer adjustment (*10*).

RESULTS

Sample Comparisons. The bulk δ¹³C for 14 of the 15 MSG trade samples analyzed indicated that they were derived from C4 carbon sources, with $\delta^{13}C_{TOT}$ ranging from −11.6 to −14.2‰ (**Table 1** and **Figure 1**). One sample (Orsan) clearly had its origin in a C3 source ($\delta^{13}C_{TOT}$ = −25.2‰).

The α-carboxyl carbons of MSG were consistently depleted in ¹³C relative to the total carbon of the molecule. Depletions ranged from −3.6 to −10.1‰ (mean ± 1 SD = −6.8 ± 1.8‰). Expressed relative to the noncarboxyl remainder of the molecule, depletions ranged from −4.5 to −12.7‰ (mean ± 1 SD = −8.5 ± 2.2‰). The magnitude of $\Delta\delta^{13}C_{RES}$ is independent of the isotopic composition of the noncarboxyl carbon but is highly correlated with the isotopic composition of the carboxyl carbon, particularly when the C4-derived samples are considered as a separate group (**Table 2**).

Individual samples could be resolved readily on the basis of $\delta^{13}C_{TOT}$. Only 10/91 possible sample pairs derived from C4

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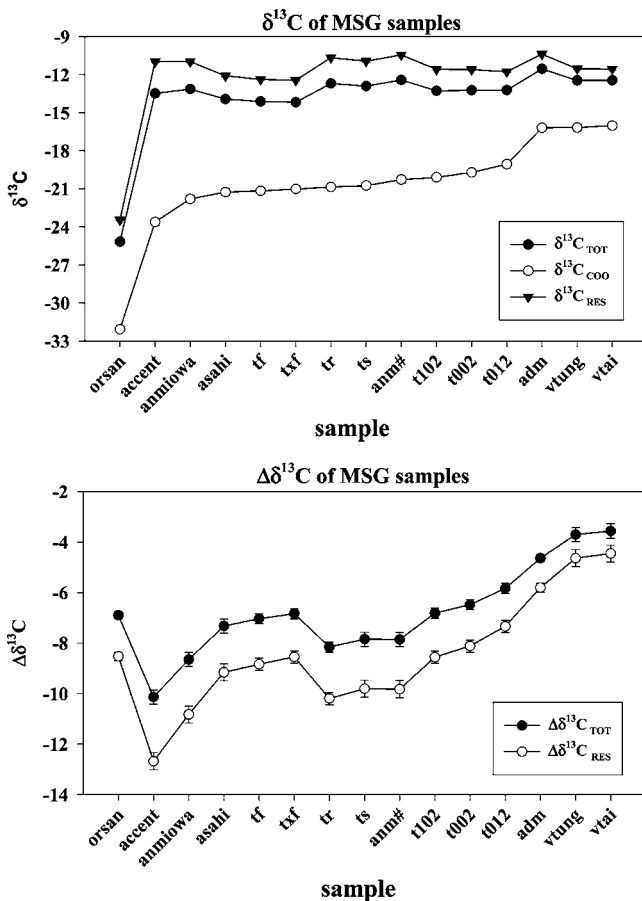
Figure 1. Plots of δ^{13}C (upper panel) or $\Delta\delta^{13}$C (lower panel) vs sample. Error bars represent one standard error. Sample codes: Orsan, Orsan; accent, (Ajinomoto) Accent; anmi, Ajinomoto Iowa; asahi, Asahi; tf, Takeda fine; txf, Takeda extra fine; TR, Takeda regular; ts, Takeda standard; anm#, Ajinomoto 150692; t102, Takeda 941102; t002, Takeda 941002; t012, Takeda 941012; adm, Archers-Daniels-Midland (ADM); vtung, Veoan Tung Hai; and vtai, Veoan Taiwan.

Table 2. Correlations of Isotopic Measurements among Samples

	$\delta^{13}C_{TOT}$	$\delta^{13}C_{COO}$	$\delta^{13}C_{RES}$	$\Delta\delta^{13}C_{TOT}$	$\Delta\delta^{13}C_{RES}$
			all samples		
$\delta^{13}C_{TOT}$	1				
$\delta^{13}C_{COO}$	0.89	1			
$\delta^{13}C_{RES}$	0.99	0.81	1		
$\Delta\delta^{13}C_{TOT}$	0.11	0.56	−0.02	1	
$\Delta\delta^{13}C_{RES}$	0.1	0.55	−0.04	0.9999	1
			C4-derived samples		
$\delta^{13}C^{TOT}$	1				
$\delta^{13}C_{COO}$	0.68	1			
$\delta^{13}C_{RES}$	0.79	0.09	1		
$\Delta\delta^{13}C_{TOT}$	0.44	0.96	−0.2	1	
$\Delta\delta^{13}C_{RES}$	0.45	0.96	−0.2	0.99998	1

carbon sources could not be distinguished. Of those, six could be resolved by $\delta^{13}C_{COO}$. The remaining five pairs (Takeda "fine"/Takeda "extra fine", Takeda "fine 941002"/ Takeda "fine 941012", Takeda "fine 941002"/Takeda "fine 941102", Takeda "fine 941012"/Takeda "fine 941102", and Veoan "Taiwan"/Veoan "Tung Hai") could not be resolved by any variable.

Manufacturer Comparisons. Because of the isotopically distinct carbon source used by Orsan to manufacture MSG, it can be readily distinguished from the other manufacturers based on its bulk isotopic composition (**Table 1**). The situation is more
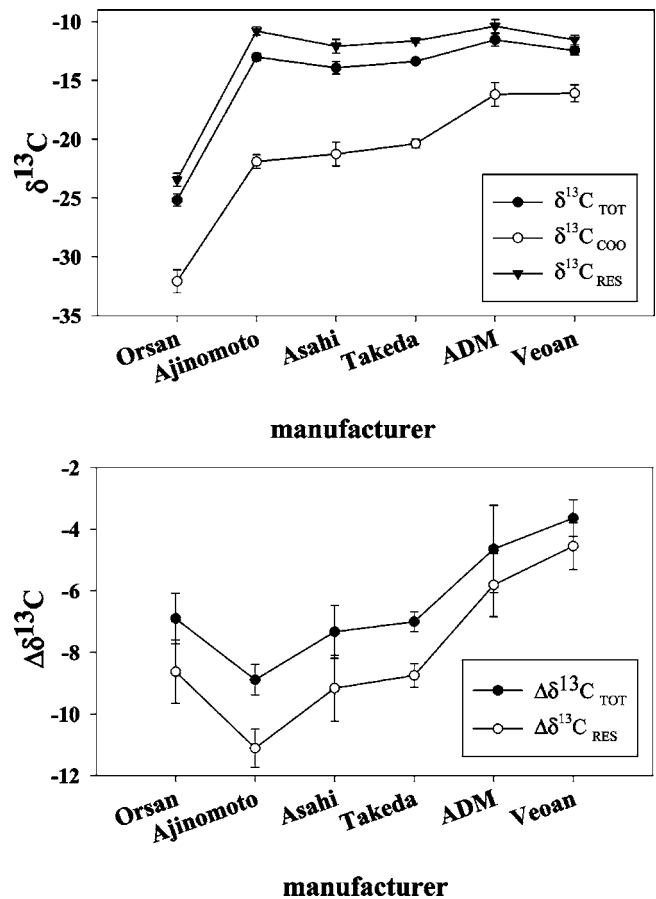
Figure 2. Plots of δ^{13}C (upper panel) or $\Delta\delta^{13}$C (lower panel) vs manufacturer. Error bars represent one standard error.

Table 3. P-values of pairwise Comparisons among Manufacturers of MSG. ANM = AJINOMOTO.

	ASAHI	ANM	TAKEDA	VEOAN	ADM
		(A) $\delta^{13}C_{TOT}$			
ORSAN	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
ASAHI		0.674	0.914	0.294	0.085
ANM			0.910	0.847	0.253
TAKEDA				0.336	0.080
VEOAN					0.730
		(B) $\delta^{13}C_{COO}$			
ORSAN	0.0001	0.0004	<0.0001	<0.0001	<0.0001
ASAHI		0.993	0.958	0.023	0.050
ANM			0.334	0.002	0.008
TAKEDA				0.005	0.030
VEOAN					1.000
		(C) $\delta^{13}C_{RES}$			
ORSAN	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
ASAHI		0.411	0.964	0.960	0.345
ANM			0.351	0.700	0.986
TAKEDA				1.000	0.383
VEOAN					0.577
		(D) $\Delta\delta^{13}C_{TOT}$			
ORSAN	0.999	0.378	1.000	0.085	0.438
ASAHI		0.631	0.999	0.053	0.298
ANM			0.081	0.001	0.015
TAKEDA				0.008	0.175
VEOAN					0.913
		(E) $\Delta\delta^{13}C_{RES}$			
ORSAN	0.999	0.377	1.000	0.012	0.438
ASAHI		0.628	0.999	0.053	0.299
ANM			0.081	0.001	0.015
TAKEDA				0.008	0.175
VEOAN					0.912

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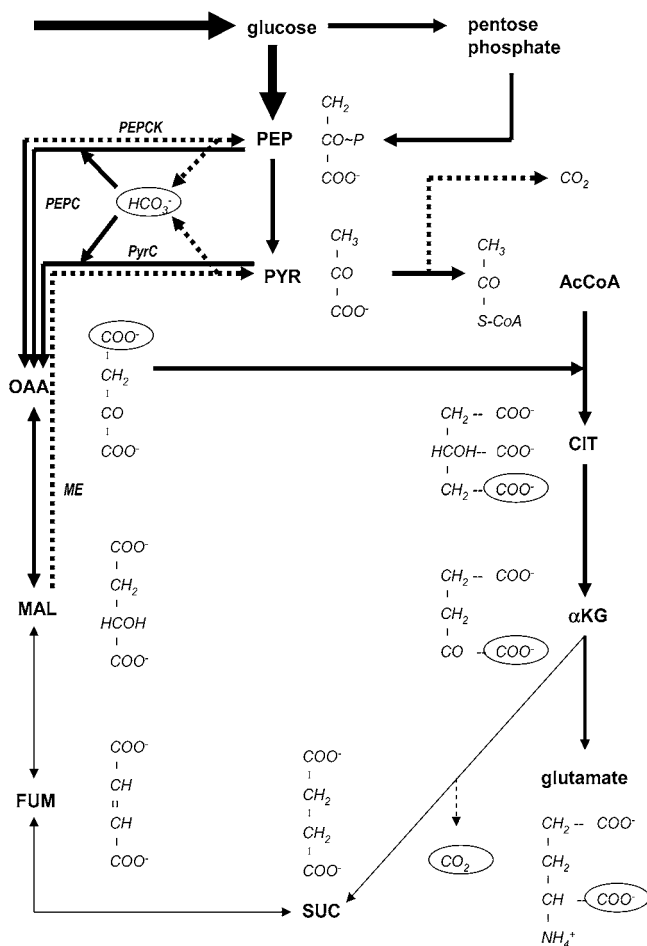
Figure 3. Metabolic fluxes during glutamate production in *Corynebacterium glutamicum.* Decarboxylating reactions noted by dashed lines. Abbreviations: AcCoA = acetyl-CoA. PEP = phosphoenolpyruvate; PYR = pyruvate, CIT = citrate; αKG = α-ketoglutarate; SUC = succinate, FUM = fumarate, MAL = malate, OAA = oxaloacetate, PEPCK = PEP carboxykinase, PyrC = pyruvate carboxylase, PEPC = PEP carboxylase, ME = malic enzyme.

complicated when the five manufacturers of MSG from the isotopically similar C4 sources are compared. At this level of analysis, no can be unambiguously identified on the basis of $\delta^{13}C_{TOT}$ alone (**Figure 2** and **Table 3**). However, 6/10 possible pairwise comparisons of $\delta^{13}C_{COO}$ among these MSG manufacturers are significantly different. Of the four remaining possible pairs, Ajinomoto and Takeda can be resolved ($p = 0.06$; C4 sources only) on the basis of $\Delta\delta^{13}C_{TOT}$ when the C4-derived samples are considered separately. The remaining sample pairs could not be resolved by any variable. Overall, inclusion of the carboxyl carbon isotope data greatly increases the possibility of resolving isotopically similar sources.

DISCUSSION

The ^{13}C depletion at the α-carboxyl carbon position of MSG is a novel observation. Previous measurements of the intramolecular isotopic compositions of amino acids in general (*11−18*) and of GLU in particular (*11, 18*) have shown that the carboxyl carbons of amino acids derived from autotrophs and multicellular heterotrophs are enriched in ^{13}C relative to bulk carbon. Unicellular microbes grown anaerobically on carbohydrate substrates have displayed GLU carboxyl carbons having isotopic compositions approximately equal to that of the bulk molecule (*11, 18*). The pattern of isotopic

enrichment in GLU carboxyl carbons within these organisms is the result of enzymatic isotope fractionations associated with anapleurotic carbon fixation and the isotopic composition of the substrate pool. The large ^{13}C depletions at the α-carboxyl carbon position in bacterially synthesized MSG can be the result of similar mechanisms and provide additional insight into the enzymatic pathways within industrial strains of corynebacteria.

Analysis of MSG from six different manufacturers allowed three isotopically distinct sources to be identified. MSG manufactured by Orsan was clearly distinguishable from the other samples on the basis of its bulk isotopic composition, indicative of a C3-derived carbon source. However, in terms of its relative ^{13}C depletion at the α-carboxyl carbon position, MSG manufactured by Orsan is not distinguishable from MSG made by Takeda, Asahi, or Ajinomoto. The other manufacturers all produce MSG from isotopically similar C4 carbon sources. Isotopic differences, where they exist, are driven almost exclusively by the relative ^{13}C depletion at the α-carboxyl carbon position.

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