Metabolic Pathway for 2-Keto-L-gulonic Acid Formation in *Gluconobacter melanogenus* IFO 3293

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The L-sorbose pathway for 2-keto-L-gulonic acid (2KGA) formation from L-sorbose in *Gluconobacter melanogenus* IFO 3293 was confirmed. L-Sorbose dehydrogenase, which catalyzes the conversion of L-sorbose to L-sorbose, was found in the membrane fraction of the microorganism, and L-sorbose dehydrogenase in the cytosol fraction. The latter enzyme requires NAD or NADP as a cofactor.

2KGA was reduced to L-idonic acid by 2KGA reductase, but L-idonic acid was re-oxidized to 2KGA by membrane-bound L-idonate dehydrogenase.

The metabolic pathway of 2-keto-L-gulonic acid (2KGA) formation from L-sorbose by *Pseudomonas putida* ATCC 21812 and *Gluconobacter melanogenus* IFO 3293 proposed by Makover *et al.*¹⁾ includes L-sorbose as an intermediate of 2KGA biosynthesis: L-sorbose $\rightleftharpoons$ L-sorbose $\rightarrow$ 2KGA. They reported the presence of 2KGA reductase, which catalyzed the conversion of 2KGA to L-idonic acid, and located L-sorbose reductase in the cytosol fraction of cell-free extract and L-sorbose dehydrogenase in the membrane fraction of *Gluconobacter melanogenus* IFO 3293. Meanwhile, Kitamura *et al.*²⁾ confirmed the pathway proposed by Makover *et al.* using a cell-free extract of the same microorganism. However, the detailed characteristics of the enzymes of this pathway have not been described.

We have reported screening for 2KGA high producers using *Gluconobacter melanogenus* IFO 3293 as a parent strain.³⁾ During the course of the screening, we obtained various metabolic mutants besides 2KGA high producers. Among them, a 2KGA non-producer, L-idonate accumulator, and L-idonate non-producer were found. Here, we measured the cellular activity of these mutants by using a

resting cell system and measured the enzyme activities in their cytosol and membrane fractions.

Materials and Methods

Microorganisms. Five mutants derived from *Gluconobacter melanogenus* SPO-1³⁾ were used. The phenotypes of these mutants are shown in Table I. All the strains were maintained on No. 4 agar medium consisting of 0.5% yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.), 0.5% glycerol, 0.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.2% agar (pH 7.0).

Chemicals. NAD, NADP, NADH, and NADPH were purchased from Oriental Yeast Industry Co., Ltd. 2,6-Dichlorophenolindophenol sodium $\cdot 2\text{H}_2\text{O}$ (DCIP) was purchased from Merck & Co., Inc. Ca-L-Idonate, L-idose, and L-sorbose were kindly supplied by Hoffmann-La Roche Inc., Nutley, N.J., U.S.A.

Table I. THE CHARACTERISTICS OF THE MUTANTS

Strain	Parent	Characteristics
UV10	SPO-1	2KGA producer
E1	D2	2KGA high producer
99-23	UV10	L-Idonate accumulator
101-25	UV10	L-Idonate non-producer
C20	SPO-1	2KGA non-producer

Analyses. Cell growth was monitored by measuring the optical density at 550 nm after dissolving the remaining CaCO_3 in the fermentation broth with the addition of 0.1 N hydrochloric acid. Analyses of the product and the substrate were made with TLC, HPLC, and GC by the procedures described previously.³⁾

Jar fermentation. Three- and five-liter jar fermentors were used to cultivate the mutants. The agar plate culture of strain E1, C20, or 99-23 was inoculated into 5 ml of medium No. 5 containing 8.0% L-sorbose, 0.05% glycerol, 1.5% yeast extract (Oriental Yeast Industry Co., Ltd.), 0.25% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.5% CaCO_3 in a test tube and cultivated at 30°C for 2 days. One ml of this culture was transferred to 50 ml of the same medium in a 500-ml Erlenmeyer flask and cultivated at 30°C for 2 days on a rotary shaker. The medium No. 3B-D containing 7.0% L-sorbose, 0.05% glycerol, 0.75% yeast extract (Difco Laboratories), 0.25% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.0% CaCO_3 was used for the strains UV10 and 101-25. The culture thus prepared was used as an inoculum for a jar fermentor containing medium No. 5 and No. 3B-D, respectively. Jar fermentors were operated under the conditions given below:

	3-l jar fermentor	5-l jar fermentor
Working volume	2 l	3 l
Agitation speed	500 rpm	300 rpm
Aeration rate	0.75 vvm	1.0 vvm
Temperature	30°C	30°C
Inoculation	2%	2%
Medium	No. 5	No. 3B-D
Cultivated mutants	E9, C20, 99-23	UV10, 101-25

After 41.5 to 65.5 hr of fermentation, the culture was harvested. The broth was centrifuged at 300 rpm for 5 min to remove calcium carbonate from the broth and at 8,000 rpm the cells were pelleted. After the cell cake was washed with 0.85% NaCl, the cells were frozen at -20°C until ready for use.

Resting cell system. For the analyses of 2KGA formation from L-sorbose, L-sorbose, and L-idonate in a resting cell system, cells of strain E1, UV10, 99-23, and C20 harvested at 41.5 hr and strain 101-25 harvested at 65.5 hr were used. Six and a half ml each of the culture broth was centrifuged at 7,000 rpm, washed twice with deionized water, and suspended in 5 ml of 0.31% NaCl solution in a test tube. One half ml of the substrate solution (30% L-sorbose or L-sorbose or 20% L-idonate) and 4.5 ml of the cell suspension were mixed and incubated at 30°C for 1 or 3 days on a test tube shaker. Cells were removed from the mixture by centrifugation after the reaction had been stopped. The amount of 2KGA, L-sorbose, and L-sorbose in the supernatant of the re-

action mixture was measured by HPLC. L-Idonate was analyzed by GC.

Preparation of cytosol and membrane fraction. Cytosol and membrane fractions were prepared in the following manner. The frozen cells (20 grams wet weight) were thawed and suspended in 10 mM potassium phosphate buffer (pH 7.0) to 50 ml. The cell suspension in the presence of glass beads (0.1 mm diameter) was homogenized by a Dyno Mill homogenizer (Willy, A. Bachofen Co., Swiss) at 2,000 rpm for 4 min at 4°C. The homogenate thus prepared was kept standing in a beaker at 4°C for 10 min and decanted to recover the supernatant. The glass beads were washed with 25 ml of the buffer and the supernatant was recovered and combined.

The supernatant thus obtained was centrifuged at 1,800 $\times g$ for 10 min to remove the cell debris, and then centrifuged at 100,000 $\times g$ for 60 min. The resulting supernatant (about 70 ml) and the precipitate are referred to here as the cytosol and membrane fractions, respectively. The membrane fraction was suspended in 35 ml of 10 mM potassium phosphate buffer (pH 7.0) and used for assays.

Enzyme assay.

1) **Cytosol enzymes.** Enzyme activity was measured by reading the change of the absorbance of reduced NAD(P) at 340 nm. The complete reaction mixture (0.41 ml) contained 1.0 mg of a substrate, 0.2 mg of NAD, NADP, NADH, or NADPH in 50 mM potassium phosphate buffer (pH 7.0), and the cytosol fraction. The reaction was started by the addition of the cytosol fraction and the change of the absorbance at 340 nm was followed at 30°C with a Kontron spectrophotometer (UVIKON 810). One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of NAD(P) or NAD(P)H per minute at 30°C.

2) **Membrane-bound enzymes.** The enzyme assay was done at room temperature by measuring the decrease in the absorbance of DCIP at 600 nm with a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the reduction of 1 μmol of DCIP per minute. The extinction coefficient of DCIP at pH 7.0 was taken as 9.45 mm^{-1} . The basal reaction mixture consisted of 3 ml of 0.1 M potassium phosphate buffer (pH 7.0), containing 0.3% Triton X-100, 0.45 ml of 2.5 mM DCIP, and 4.95 ml of water. This was prepared just before the assay. A cuvette with a 1-cm light path contained 0.4 ml of basal mixture, 5–100 μl of 1 M substrate, enzyme solution, and water in a final volume of 0.51 ml. A reference cuvette contained all components except the substrate. Enzyme activity was measured as the initial reduction rate of DCIP.

Results

Preparation of cells

The cell used for the resting cell system and

Table II. ANALYTICAL DATA ON THE CULTURE BROTH OF THE MUTANTS

Strain	Cultivation time (hr)	OD ₅₅₀	pH	2KGA (g/l)	L-Idonate (g/l)	Cells recovered (wet weight, g)
UV10	41.5	24.2	5.49	4.94	16.6	58.6
E1	41.5	35.9	5.54	17.14	14.0	45.8
99-23	41.5	33.2	5.37	4.40	26.7	40.0
101-25	65.5	12.4	5.34	16.9	1.6	21.0
C20	41.5	28.8	5.38	0	0	31.4

Table III. IDENTIFICATION ON THE PRODUCTS FROM SEVERAL SUBSTRATES BY A RESTING CELL SYSTEM

Substrate	Product	UV10	E1	99-23	101-25	C20
L-Sorbose	2KGA	+	+	+	+	—
	L-Idonate	+	+	+	—	—
L-Sorbose	2KGA	+	+	+	+	+
	L-Idonate	+	+	+	—	+
L-Idonate	2KGA	+	+	—	+	+

+, produced: —, not produced.

enzymatic analyses were prepared by using 3- or 5-l jar fermentation as described in Materials and Methods. The analytical data on the culture broth are summarized in Table II. Because the growth of strain 101-25 was slower than the other strains, this culture was harvested after 65.5 hr. For the strain 101-25, the recovered cell mass was much less compared with all the others. Because the fermentation was stopped during the 2KGA production phase, the amount of 2KGA produced at the time of harvest did not reflect the complete 2KGA productivity of the mutants. It is assumed that all the strains were harvested in the middle logarithmic phase of their growth.

Resting cell system

We used a resting cell system to examine the cellular activity for 2KGA formation using L-sorbose, L-sorbose, and L-Idonate as substrates by the procedure described in Materials and Methods. As shown in Table III, all the strains except for C20 produced 2KGA from L-sorbose and strain 101-25 did not accumulate L-Idonate from L-sorbose or L-sorbose. All the strains produced 2KGA

Table IV. COMPARISON OF 2KGA PRODUCTIVITY OF A RESTING CELL SYSTEM OF THE MUTANTS AT THE SAME CONCENTRATION OF CELLS

Strain	Substrate						
	L-Sorbose		L-Sorbose		L-Idonate		
	Day	1	3	1	3	1	3
UV10		3.4 ^a	7.9	5.3	7.2	1.4	2.5
E1		9.2	27.1	6.3	11.0	7.4	11.9
99-23		5.7	11.6	6.4	9.7	0	0
101-25		23.8	46.4	13.8	22.7	trace	2.4
C20		0	0	3.4	6.9	1.5	2.6

The cell concentration was adjusted at 30.0 at OD_{550 nm}.

^a 2-KGA (grams per liter).

from L-sorbose. Strain 99-23 did not produce 2KGA from L-Idonate, while the other strains did. It should be noted that strain 99-23 which was selected as a L-Idonate accumulator is deficient in the conversion of L-Idonate to 2KGA.

2KGA productivity of the mutants at the same cellular concentrations was compared. The results are shown in Table IV. Among them, strain 101-25 had the highest 2KGA

productivity per cell from L-sorbose and L-sorbose. The E1 strain produced the largest amount of 2KGA from L-idonate.

Table V. PROTEIN CONCENTRATION OF THE CYTOSOL FRACTION

Strain	Protein concentration (mg/ml)
UV10	44.3
E1	42.9
99-23	43.2
101-25	44.7
C20	51.0

Enzymatic study

1) *Cytosol enzymes.* At the protein concentrations of each cytosol listed in Table V, we measured various enzyme activities. As shown in Table VI, all the strains had rather high NADH oxidase activity. Therefore, when we calculated the enzyme activity when NADH was the cofactor, we subtracted the NADH oxidase activity from the apparent activity.

We suggest the presence of NADPH-dependent L-sorbose reductase, NAD and/or NADP-dependent L-sorbose dehydrog-

Table VI. ENZYME ACTIVITIES IN THE CYTOSOL FRACTION

Mutant	Substrate								
	L-Sorbose		L-Sorbose			2-Keto-L-gulonic acid		None	
	Cofactor	NADH NADPH	NAD NADP NADPH			NADH NADPH			NADH NADPH
UV10		5.6 22.1 (100) (100)	14.7 9.3 20.3 (100) (100) (100)			18.5 69.8 (100) (100)			11.1 1.8 (100) (100)
E1		0 21.0 (0) (95)	49.2 22.8 11.4 (335) (245) (56)			11.4 94.6 (62) (136)			15.4 1.9 (139) (106)
99-23		3.7 15.3 (66) (69)	41.4 17.1 9.5 (282) (184) (47)			5.8 71.5 (31) (102)			11.3 1.9 (102) (106)
101-25		3.6 14.8 (64) (67)	61.7 29.3 16.6 (420) (315) (82)			0 7.2 (0) (10)			29.3 3.6 (264) (200)
C20		3.1 24.1 (55) (109)	31.8 17.6 30.6 (216) (189) (151)			8.0 111.4 (43) (160)			21.0 3.1 (189) (172)

Enzyme activity: m Unit/mg protein. Relative activity is shown in the parenthesis.

Table VII. CONVERSION PRODUCTS OF SEVERAL SUBSTANCES BY THE CYTOSOL FRACTION

Mutant	Products detected on TLC			
	Substrate			
	L-Sorbose	L-Sorbose	2-Keto-L-gulonic acid	
UV10	D-Sorbitol (+ + +)	L-Sorbose (+ +) L-Idonate (+)	2KGA (+ +)	None
E1	D-Sorbitol (+ + +)	L-Sorbose (+ +) L-Idonate (+)	2KGA (+ +)	None
99-23	D-Sorbitol (+)	L-Sorbose (+ +) L-Idonate (+)	2KGA (+ +)	None
101-25	D-Sorbitol (+ + +)	L-Sorbose (+ ~ + +)	2KGA (+ + +)	None
C20	D-Sorbitol (+ + +)	L-Sorbose (+ +) L-Idonate (+)	2KGA (+ + +)	L-Idonate (±)

+ ~ + + + : The quantity of the product is figured.

enase, NADPH-dependent L-sorbose reductase, and NADPH-dependent 2KGA reductase in the cytosol fraction of *Glucobacter melanogenus* IFO 3293. In fact, we have already isolated two of these enzymes, L-sorbose reductase and L-sorbose dehydrogenase and strongly suggest that L-sorbose dehydrogenase found in the cytosol fraction is predominantly active in the conversion of L-sorbose to 2KGA, because the membrane-bound L-sorbose dehydrogenase was less active in this microorganism, as described later. Strain E1, a 2KGA high producer, had about three-fold higher L-sorbose dehydrogenase activity than strain UV10 had.

Strain 101-25, which is a L-idonate non-producer, has little or almost no 2KGA reductase activity. This means that L-idonate is mainly produced from 2KGA. All strains except for 101-25 had very high 2KGA reductase activity, which was stronger than L-sorbose dehydrogenase activity. It is likely that 2KGA formed in the cytosol immediately can be converted to L-idonate by the reductase reaction, if the NADPH concentration is high enough.

Using TLC analysis, we simultaneously checked the conversion of the products of several compounds in the cytosol fraction in the absence of exogenous cofactor. The results are shown in Table VII. The reduction of 2KGA to L-idonate was done when L-sorbose was a substrate. Nevertheless, L-idonate was formed from 2KGA when exogenous NADPH was added to the reaction mixture (data not shown). We suggest that NADP in the cytosol was present in its oxidative form, and NADPH was formed in accordance with L-sorbose oxidation to 2KGA and could sequentially serve the 2KGA reduction. Formation of D-sorbitol from L-sorbose, and of L-sorbose from L-sorbose was observed in all the cases tested.

2) *Membrane-bound enzymes.* Various substrates were reacted with the crude membrane-bound enzyme preparation for 4 hr at 30°C in the presence of phenazine methosulfate as an artificial electron acceptor. Products were analyzed on TLC and HPLC. The results are shown in Table VIII. 2KGA formation was commonly detected from L-sorbose, L-idonate, L-idose, and D-sorbitol by all the strains except for strains C20 and 99-23. Strains C20 and 99-

Table VIII. REACTION PRODUCTS BY MEMBRANE-BOUND ENZYME

Mutant	Substrate					
	D-Sorbitol	L-Sorbose	L-Sorbose	2KGA	L-Idonate	L-Idose
UV10	L-Sorbose (+++) L-Sorbose (+) 2KGA (+)	L-Sorbose (+) 2KGA (±)	2KGA (+)	None	2KGA (+++)	2KGA (+)
E1	L-Sorbose (+++) L-Sorbose (+) 2KGA (+)	L-Sorbose (+) 2KGA (+)	2KGA (+)	None	2KGA (+++)	2KGA (+)
99-23	L-Sorbose (+++) L-Sorbose (+) 2KGA (+)	L-Sorbose (+) 2KGA (±)	2KGA (+)	None	None	2KGA (+)
101-25	L-Sorbose (+++) L-Sorbose (+) 2KGA (+)	L-Sorbose (+) 2KGA (±)	2KGA (+)	None	2KGA (+++)	2KGA (+)
C20	L-Sorbose (+++)	None	2KGA (+)	None	2KGA (+++)	2KGA (+)

± ~ + + +: The quantity of the product is figured.

Table IX. SUBSTRATE SPECIFICITY OF MEMBRANE FRACTION OF THE MUTANTS (DCIP METHOD)

Strain	Protein conc. (mg/ml)	Enzyme activity [U/mg-protein] on				
		D-Sorbitol	L-Sorbose	2KGA	L-Idonate	L-Idose
UV10	4.4	0.074 (100)	0.28 (100)	0	0.62 (100)	0.089 (100)
E1	4.2	0.077 (104)	0.42 (150)	0	1.24 (201)	0.091 (102)
99-23	6.75	0.019 (26)	0.16 (56)	0	0 (0)	0.065 (73)
101-25	7.8	0.033 (45)	0.21 (74)	0	0.52 (85)	0.049 (55)
C20	7.7	0.072 (97)	0 (0)	0	0.88 (143)	0.053 (60)

(): Relative activity.

23, deficient in L-sorbose dehydrogenase and L-idonate dehydrogenase, respectively, did not convert L-sorbose and L-idonate to 2KGA, respectively. 2KGA was not metabolized by the membrane-bound enzyme preparation. From these observations, we suggest that *G. melanogenus* IFO 3293 has D-sorbitol dehydrogenase, L-sorbose dehydrogenase, L-sorbose dehydrogenase, L-idonate dehydrogenase, and L-idose dehydrogenase in its membrane fraction.

Various enzyme activities of the membrane fraction on different substrates were examined by the DCIP method. The results are summarized in Table IX. The enzyme activity of membrane-bound L-sorbose dehydrogenase could not be measured with the DCIP method because L-sorbose itself strongly decolorized DCIP in the reaction mixture. The strain E1, a 2KGA high producer, showed about 1.5 times higher L-sorbose dehydrogenase activity than UV10. The high L-idonate dehydrogenase activity of the strain E1 was notable as well. Deficiency of L-sorbose dehydrogenase in the strain C20 and of L-idonate dehydrogenase in the strain 99-23 was confirmed enzymatically.

3) *Comparison of 2KGA productivity from L-sorbose using the cytosol and the membrane fractions of the strain E1.* As described above,

Table X. COMPARISON OF 2KGA PRODUCTIVITY FROM L-SORBOSONE USING THE CYTOSOL AND THE MEMBRANE FRACTIONS OF THE STRAIN E1

Source	Product (g/l)	
	2KGA	L-Idonate
Cytosol fraction	1.75	1.65
Membrane fraction	0.1	0

The reaction mixture containing 0.2ml of 0.5M potassium phosphate buffer (pH 7.0), 0.2ml of 0.5M L-sorbose solution, 1.0ml of water and 0.4ml of the cytosol or the membrane fraction was incubated at 30°C for 5 hr with shaking. Amounts of 2KGA and L-idonate were measured by HPLC and GC, respectively.

two types of L-sorbose dehydrogenases: cytosol and membrane-bound types, are present in *G. melanogenus* IFO 3293. To compare both enzyme activities for 2KGA formation from L-sorbose, a product assay was done because the photometric assay did not work for membrane-bound L-sorbose dehydrogenase. Concentrations of the cytosol and the membrane fractions used for the assay were adjusted so that their ratio might be nearly the same as that in an intact cell. The results are shown in Table X. In the assay using the cytosol fraction, 1.75 g of 2KGA per liter and

1.65 g of L-idonate per liter were produced. On the other hand, 0.1 g of 2KGA per liter was produced by the membrane fraction. Since L-idonate formed in the cytosol fraction was considered to be derived from 2KGA through the reductive reaction by 2KGA reductase, the total amount of 2KGA formed by the cytosol fraction was roughly calculated to be about 3.4 g per liter. Consequently, cytosol L-sorbose dehydrogenase was about 30 times more active than membrane-bound L-sorbose dehydrogenase.

Discussion

From the results of enzymatic analyses of the mutants, we conclude that strains C20, 99-23, and 101-25 are blocked mutants of L-sorbose dehydrogenase, L-idonate dehydrogenase, and 2KGA reductase, respectively. Accordingly, strain C20 is unable to produce 2KGA from L-sorbose, but is able to produce it from L-sorbose. Because strain 99-23 does not have L-idonate dehydrogenase, the accumulation of L-idonate was observed in the fermentation broth. Strain 101-25 does not accumulate L-idonate in the culture broth from L-sorbose, and its 2KGA reductase is blocked. This strongly suggests that 2KGA is a main precursor of L-idonate in the fermentation process. High 2KGA reductase activity was found in all the strains except for strain 101-25.

The 2KGA high producer, strain E1, had higher L-sorbose dehydrogenase, L-sorbose dehydrogenase, and L-idonate dehydrogenase activities. This indicated that the improvement in 2KGA productivity from L-sorbose of this

strain was a result of the increase in quantity or the change in quality of the several enzymes concerned, not because of one particular enzyme.

When the enzymatic activity to convert L-sorbose or L-sorbose to 2KGA of the strains was compared at the same cell concentration of a resting system, strain 101-25 had the highest conversion activity. It is characteristic for this strain to have high L-sorbose dehydrogenase activity, but this strain grew so slowly in the production medium and so much L-sorbose remained that the production of 2KGA was not feasible.

In summary, D-sorbitol, L-sorbose, and L-idonate dehydrogenases of *Gluconobacter melanogenus* IFO 3293 are located in the membrane fraction of the cell and L-sorbose reductase and 2KGA reductase are located in the cytosol fraction of the cell. L-Sorbose dehydrogenase is mainly found in the cytosol fraction. Accordingly, we suggest that the permeation of L-sorbose, which is generated outside the cell, into the cytosol may be important for the formation of 2KGA. To produce 2KGA continuously from L-sorbose, regeneration of NADH or NADPH might be necessary. L-Sorbose reductase, L-sorbose reductase, 2KGA reductase, and NAD(P)H oxidase may function for this purpose. L-Idonate formed could be easily converted to 2KGA again by the membrane-bound L-idonate dehydrogenase.

Kitamura *et al.*²⁾ reported that L-sorbose and L-sorbose dehydrogenases of *Gluconobacter melanogenus* IFO 3293 were mainly located in the membrane fraction. In our ob-

Fig. 1. Proposed Mechanism of 2KGA-relating Pathways of *Gluconobacter melanogenus* IFO 3293

Abbreviations for enzymes: SLDH, D-sorbitol dehydrogenase; SDH, L-sorbose dehydrogenase; SNDH, L-sorbose dehydrogenase; IDH, L-idonate dehydrogenase; SR, L-sorbose reductase; SNR, L-sorbose reductase; 2KR, 2-keto-L-gulonate reductase.

servation, L-sorbose dehydrogenase is located in the membrane fraction, but L-sorbose dehydrogenase is mainly located in the cytosol fraction. We consider that membrane-bound L-sorbose dehydrogenase must be present to a minor extent.

Based on our observations, we illustrate in Fig. 1 the presumptive metabolic linkage of sugars relating to 2KGA formation of *Gluco-*

nobacter melanogenus IFO 3293.

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