Biochimica et Biophysica Acta, 614 (1980) 256—265

BBA 69064

PURIFICATION AND PROPERTIES OF NAD$^+$-DEPENDENT MALTOSE DEHYDROGENASE PRODUCED BY ALKALOPHILIC *CORYNEBACTERIUM* sp. NO. 93-1

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(Received November 12th, 1979)
(Revised manuscript received March 27th, 1980)

Key words: NAD$^+$ dependence; Maltose dehydrogenase; Alkalophile; (Corynebacterium sp. No. 93-1)

Summary

NAD$^+$-dependent maltose dehydrogenase was purified about 250-fold from the cell free extract of an alkalophilic *Corynebacterium* sp. No. 93-1. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and ultracentrifugation. The molecular weight of the enzyme was determined to be 40 000 ± 2000 by gel filtration and SDS-polyacrylamide gel electrophoresis. The enzyme appeared to be a single peptide chain. The isoelectric point was pH 4.50. The optimal pH was 10.2. The enzyme was stable over the range of pH 6 to 10. NAD$^+$-dependent maltose dehydrogenase showed very wide substrate specificity on monosaccharides, disaccharides and trisaccharides. Among these substrates, maltose was the most reactive. Also, the enzyme showed oxidative activity on maltotetraose and maltopentaose. The K_m values at pH 10 were 2.1 mM for maltose and 0.15 mM for NAD$^+$. It was conjectured that the primary product of this reaction was maltono-δ-lactone and it was hydrolyzed non-enzymatically to maltobionic acid. *p*-Chloromercuribenzoic acid, Hg^{2+} and Ag^{2+} completely inhibited the activity, and NADH also showed competitive inhibition on the activity.

Introduction

Sugar dehydrogenase systems of genus *Pseudomonas*, *Gluconobacter* and *Bacillus* were examined in detail [1—9]. But as regards carbohydrate metab-

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olism of coryneform bacteria, studies were carried out mainly from a viewpoint of glutamic acid production [10—11]. Up to now, as far as we know, there are few reports on a sugar oxidation system of coryneform bacteria.

In a previous paper [12], we have reported on the isolation and identification of an alkalophilic *Corynebacterium* sp. No. 93-1. The crude extract of this strain contained three kinds of $NAD^+(P^+)$-dependent dehydrogenase activity which reacted on maltose and D-glucose. They were NAD^+-dependent maltose dehydrogenase, $NADP^+$-dependent maltose dehydrogenase and NAD^+-dependent D-glucose dehydrogenase. The characteristics of these three kinds of dehydrogenase were interesting from a standpoint of comparison with already reported sugar dehydrogenases. This paper deals with the purification and properties of NAD^+-dependent maltose dehydrogenase.

Materials and Methods

Culture. An alkalophilic *Corynebacterium* sp. No. 93-1 was cultured as described previously [12].

Enzyme assay. NAD^+-dependent maltose dehydrogenase, $NADP^+$-dependent maltose dehydrogenase and NAD^+-dependent D-glucose dehydrogenase activities were assayed by the methods described in a previous paper [12].

Polyacrylamide gel electrophoresis. Electrophoresis on polyacrylamide gel was carried out at the running pH 8.0 using 7.5% concentration of acrylamide. Electrophoresis was performed at 3 mA/tube at 5°C. Protein was stained with Coomassie brilliant blue R250. NAD^+-dependent maltose dehydrogenase activity was detected by the method of Pauly and Pfleiderer [9] with a slight modification (pH 9.0).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Electrophoresis was performed according to Weber and Osborn [13], using 10% acrylamide gel containing 0.1% SDS. Sample was treated with 5% 2-mercaptoethanol and 1% SDS in 10 mM phosphate buffer (pH 7.2) for 2 h at 50°C. Electrophoresis was carried out at 8 mA/tube at 10°C.

Isoelectric focusing. The isoelectric point was estimated using an isoelectric focusing method. It was performed using 1% (v/v) Ampholine (pH 4—6) and sucrose gradient in a 110 ml column (LKB Produkters) at 1000 V for 15 h at 2°C.

Gel filtration. The determination of the molecular weight of the enzyme was done according to Andrew [14], using a column (1.6 × 100 cm) of Ultrogel AcA44 equilibrated with 50 mM phosphate buffer (pH 7.5) containing 10% (v/v) glycerol and 1 mM 2-mercaptoethanol.

Ultracentrifugation. The sedimentation experiment was carried out by a Spinco Model E ultracentrifuge (Beckman Instrument). The sedimentation coefficient, $s_{20,w}$, was determined.

Thin-layer chromatography. The analysis of the product from maltose by NAD^+-dependent maltose dehydrogenase was carried out on TLC using Avicel SF (Funakoshi Co., Ltd.) plates. The solvent systems were as follows; solvent A, ethyl acetate/pyridine/acetic acid/water (5 : 5 : 1 : 3, v/v), solvent B, *n*-butanol/pyridine/water (1 : 1 : 1, v/v) and solvent C, isopropanol/water (4 : 1, v/v). Sugar acid was detected by spraying with a mixture of bromo-

phenol blue and methyl orange solution. The detection of keto-sugar was carried out by the method of Lanning and Cohen [15].

Protein determination. Protein was measured from absorbance at 260 and 280 nm according to Layne [16].

Chemicals. NAD^+, $NADP^+$, NADH and NADPH were purchased from Kyowa Hakko Kogyo Co., Ltd. Maltose was from Nakarai Chemical Co., Ltd. Maltotriose, maltotetraose and maltopentaose were gifts from Dr. N. Nakamura of Nihon Shokuhin Kako Co., Ltd. Maltobionic acid was also a gift from Dr. K. Watanabe of our laboratory. All other compounds used were of reagent grade.

Purification. The results of the purification are summarized in Table I.

Step 1. Extraction. 80 g (wet weight) of the 24 h cultured cells were subjected to sonic oscillation in 20 mM phosphate buffer (pH 7.3), containing 5 mM 2-mercaptoethanol, 1 mM EDTA and phenylmethylsulfonyl fluoride (2 μg/ml) at 20 KHz. The crude extract was obtained by centrifugation at 20 000 × *g* for 20 min.

Step 2. Streptomycin sulfate treatment. After dilution with water, 5% streptomycin sulfate solution was added to the crude extract dropwise. The precipitate was removed by centrifugation and the supernatant solution was obtained.

Step 3. $(NH_4)_2SO_4$ fractionation. The precipitate at 30—70% $(NH_4)_2SO_4$ was prepared and dissolved in a minimal volume of 50 mM phosphate buffer (pH 7.3) containing 10% (v/v) glycerol/1 mM 2-mercaptoethanol/1 mM EDTA/ 1 mM phenylmethylsulfonyl fluoride (buffer A) and dialyzed overnight against the same buffer.

Step 4. DEAE-cellulose column chromatography. The enzyme solution was loaded onto a DEAE-cellulose column (9 × 40 cm) equilibrated with buffer A. The enzyme was eluted with a linear gradient of NaCl (0—0.8 M). As shown in Fig. 1, the peaks of these three enzymes activities were separated.

Step 5. Gel filtration on Ultrogel AcA44. The concentrated enzyme solution was placed on an Ultrogel AcA44 column (2.5 × 100 cm) and eluted with buffer A. The active fractions were pooled and dialyzed against 10 mM phosphate bffer (pH 7.3), containing 10% (v/v) glycerol, 1 mM 2-mercaptoethanol and 1 mM EDTA (buffer B).

TABLE I

SUMMARY OF THE PURIFICATION OF NAD^+-DEPENDENT MALTOSE DEHYDROGENASE

	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification (-fold)
Crude extract	300	933	7560	0.123	100	1
Streptomycin sulfate treatment	970	831	3735	0.222	89.1	1.80
$(NH_4)_2SO_4$ fractionation	212	698	2078	0.336	74.8	2.73
DEAE-cellulose	222	445	422	1.05	48.8	8.54
Ultrogel AcA44	40	381	30.5	12.3	40.8	100
Hydroxyapatite	71	329	17.0	19.4	35.3	158
DE-52 cellulose	18	290	10.5	26.4	31.1	215
Biogel P-100	21	248	7.93	31.0	26.6	252

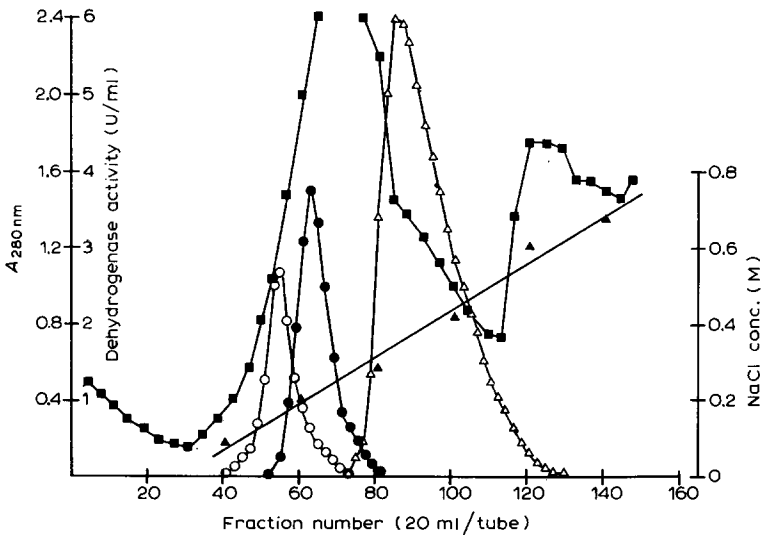

Fig. 1. Elution pattern of NAD^+-dependent maltose dehydrogenase, $NADP^+$-dependent maltose dehydrogenase and NAD^+-dependent D-glucose dehydrogenase on DEAE-cellulose column chromatography. The enzymes' activities were assayed under the standard assay conditions. NAD^+-dependent maltose dehydrogenase (○———○), $NADP^+$-dependent maltose dehydrogenase (●———●), NAD^+-dependent D-glucose dehydrogenase (△———△), O.D. 280 nm (■———■), NaCl (▲———▲).

Step 6. Hydroxyapatite column chromatography. The enzyme solution was applied to a hydroxyapatite column (2.5 × 17 cm) equilibrated with buffer B. The enzyme was eluted with a linear gradient of 10—200 mM phosphate buffer (pH 7.3) (the same contents as buffer B). The active fractions were dialyzed against buffer C (except phenylmethylsulfonyl fluoride from buffer A).

Step 7. DE-52 cellulose column chromatography. The enzyme solution was applied to a DE-52 cellulose column (1.2 × 18 cm) equilibrated with buffer C. The enzyme was eluted with a linear gradient of NaCl (0—0.5 M).

Step 8. Gel filtration on Biogel P-100. The concentrated enzyme solution was passed through a Biogel P-100 column (2.0 × 90 cm) equilibrated with buffer C. The active fractions were pooled and concentrated using a Diaflow membrane PM-10. This enzyme solution was used for the following experiments.

Results

Homogeneity

The homogeneity of the purified enzyme was investigated using three methods; polyacrylamide gel electrophoresis, SDS polyacrylamide gel electrophoresis and ultracentrifugal analysis. As shown in Fig. 2, the purified enzyme gave a single protein band on polyacrylamide gel. The sample also gave a single band on SDS polyacrylamide gel. Ultracentrifugal analysis indicated that the preparation was completely purified and $s_{20,w}$ was 3.6 S.

Physical properties

(1) Molecular weight. The molecular weight of the enzyme was estimated by

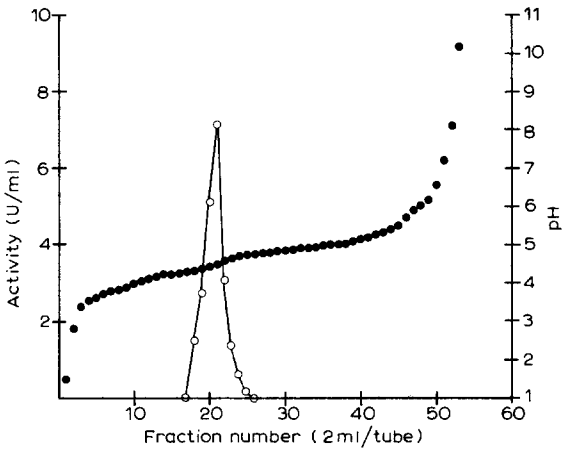

Fig. 2. Polyacrylamide gel electrophoresis of NAD^+-dependent maltose dehydrogenase. A, protein stain; B, activity stain. About 30 μg of the sample were used.

Fig. 3. Determination of the isoelectric point of NAD^+-dependent maltose dehydrogenase. About 30 units of the enzyme were used and the activity was assayed under the standard assay conditions. NAD^+-dependent maltose dehydrogenase activity (○———○); pH (●———●).

the gel filtration method and using SDS polyacrylamide gel electrophoresis. The molecular weight of 40 000 ± 2000 was found using these two methods. From these results, it was suggested that the enzyme was composed of a single peptide chain.

(2) Isoelectric point. The isoelectric point of the enzyme was investigated by the isoelectrofocusing method. The preparation showed a single peak of activity and the isoelectric point was pH 4.50 (Fig. 3).

(3) Ultraviolet spectrum. The enzyme showed the absorption spectrum of a typical protein with no evidence of any prosthetic group. The absorption maximum was 280 nm and the minimum 251 nm. The absorption ratio 280 nm/260 nm was 1.67 at pH 7.2.

Effect of pH

The enzyme activity was determined at various pH values. The optimal pH for the enzyme activity was around 10.2 in 50 mM glycine/NaOH buffer. Also, pH stability was investigated at various pH values. Fig. 4 shows that the enzyme was stable within the wide range pH 6—10.

Substrate specificity

Substrate specificity of the enzyme was investigated and the results were summarized in Table II. The enzyme showed very wide specificity from monosaccharides to pentasaccharide such as maltopentaose. Among these reactive substrates, maltose was the most effective. As regards cofactors, NAD^+ was more effective than $NADP^+$ on all reactive substrates.

TABLE II

SUBSTRATE SPECIFICITY OF NAD^+-DEPENDENT MALTOSE DEHYDROGENASE

The assay was carried out using the standard assay method using 100 mM of substrates (Section A) and 10 mM of substrates (Section B). The relative reaction velocities for various kinds of substrate were related to that of maltose as 100% with NAD^+ as a cofactor.

Substrate	Section A					Section B		
	Relative rate		Substrate	Relative rate		Substrate	Relative rate	
	NAD^+	$NADP^+$		NAD^+	$NADP^+$		NAD^+	$NADP^+$
D-Glucose	63	3	D-Xylitol	0	0	D-Glucose	28	0
D-Mannose	6	0	D-Sorbitol	0	0	Maltose	100	1
D-Galactose	85	3	D-Arabinitol	7	0	Maltotriose	100	1
D-Fructose	44	22	Inositol	62	0	Maltotetraose	75	0
D-Xylose	55	4	L-Rhamnose	85	1	Maltopentaose	65	0
D-Ribose	16	5	L-Fucose	62	0			
D-Arabinose	84	0	2-Deoxy-D-Glucose	17	10			
Maltose	100	10	Raffinose	18	1			
Lactose	91	9	Melezitose	0	0			
Cellobiose	84	6	Trehalose	0	0			
Melibiose	45	9	Glucose-6-*P*	6	0			
Sucrose	0	0	D-Glucosamine	0	0			
D-Mannitol	0	0	NAc-D-Glucosamine	15	0			

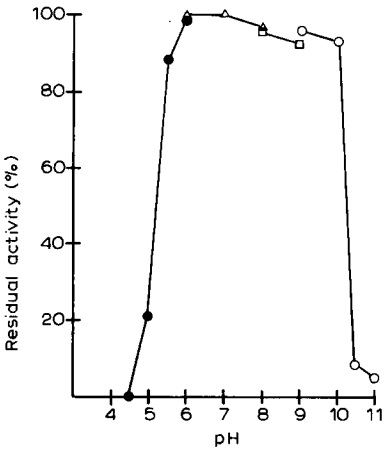

Fig. 4. pH stability of NAD^+-dependent maltose dehydrogenase. After the treatment of the purified enzyme in each buffer for 30 min at 30°C, the residual activity was measured. The buffer system was as follows; pH 4.5—6, 100 mM succinate buffer (●———●); pH 6—8, 100 mM phosphate buffer (△———△); pH 8—9, 100 mM Tris-HCl buffer (□———□); pH 9—11, 100 mM glycine-NaOH buffer (○———○).

Effect of the concentration of substrates and NAD^+

Estimation of the K_m and V values for six kinds of substrate was performed by the Lineweaver-Burk plot at pH 10.0. Also, in regard to maltose, the same analysis was carried out at pH 8.0. Results obtained are listed in Table III. It was clear that the enzyme showed the highest affinity for lactose. The effect of the concentration of NAD^+ on the enzyme activity was examined using 100 mM maltose at pH 10.0. The K_m value was 0.15 mM.

Analysis of the product

A product produced from maltose by the enzyme was analyzed by TLC using Avicel SF plates with solvent A, B and C. A yellow spot corresponding to maltobionic acid was detected. The R_f values were 0.19 on solvent A, 0.33 on solvent B and 0.20 on solvent C. But no keto-sugar was found on any plate. On the basis of these results, it was expected that the primary product was

TABLE III

KINETIC CONSTANTS OF NAD^+-DEPENDENT MALTOSE DEHYDROGENASE

K_m and V values for six kinds of substrate were estimated by Lineweaver-Burk analysis at pH 10.0.

Substrate	K_m (mM)	V (μmol/min per mg protein)
D-Glucose	23	20.0
D-Galactose	3.0	25.5
Maltose (at pH 8.0)	13	13.0
(at pH 10.0)	2.1	31.0
Lactose	0.49	25.9
Cellobiose	13	22.4
Maltotriose	1.4	26.7

maltono-δ-lactose and it was hydrolyzed non-enzymatically to maltobionic acid.

Effect of various kinds of reagent on the activity

The effect of the various kinds of salt, inhibitor, metal ion, nucleotide and sugar phosphate on the activity was examined. The activity was completely inhibited by 0.1 mM of *p*-chloromercuribenzoic acid, 1 mM of Hg^{2+} or Ag^{2+}. NADH (0.2 mM) inhibited the activity about 50%, 0.2 mM NADPH or 3 mM NADP weakly inhibited the activity. Nucleotides such as AMP, ADP, ATP and cyclic AMP had no effect. And intermediates of glycolysis such as Glc-6-*P*, Fru-6-*P*, Fru-1,6-P_2 and 6-phosphogluconate also did not act as activators or inhibitors.

Effect of NADH on the enzyme activity

The effect of NADH on the enzyme activity was examined by Lineweaver-Burk analysis using NAD^+ (3 mM) or maltose (100 mM) as a saturated substrate. NADH acted as a competitive inhibitor of maltose or NAD^+ and K_i values for maltose and NAD^+ were 0.042 mM and 0.023 mM, respectively 5A and B).

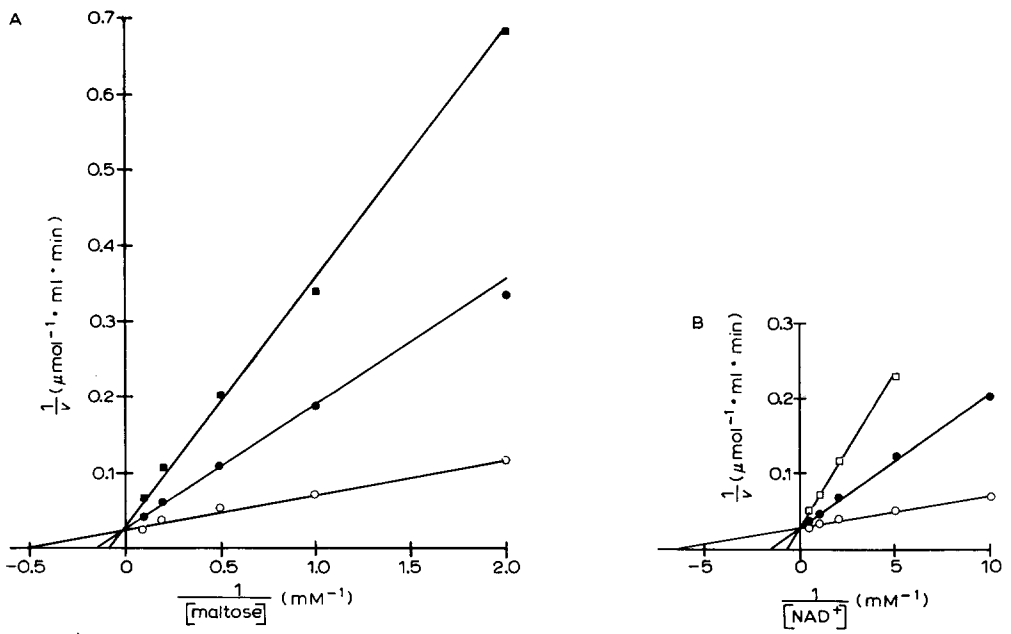

Fig. 5. Lineweaver-Burk analysis of the effect of NADH on NAD^+-dependent maltose dehydrogenase. A, the effect was investigated under the condition that NAD^+ was saturated (3 mM). NADH concentrations were 0.1 mM (●———●) and 0.2 mM (■———■). Control was assayed in the standard assay method (○———○). The enzyme activity used was 0.041 units. B, the same analysis was carried out under the condition that maltose was saturated (100 mM). NADH concentrations were 0.1 (●———●) and 0.2 mM (■———■). Control was assayed in the standard assay method (○———○). The enzyme activity used was 0.015 units.

Discussion

The direct oxidation system of disaccharides (e.g. lactose, maltose, cellobiose) to the corresponding aldobionic acids by genus *Pseudomonas* is well known. Nishizuka and Hayaishi [17] reported about lactobionic acid formation from lactose by *Pseudomonas graveolens* and they purified corresponding lactose dehydrogenase. The enzyme showed very wide substrate specificity on monosaccharides and disaccharides (e.g. δ-lactose, β-lactose, maltose, cellobiose). The enzyme could use artificial electron acceptors but could not use NAD^+ and $NADP^+$. Cuatrecasas and Segel [18] described NAD^+-dependent D-galactose dehydrogenase from rat liver. This enzyme also showed very broad substrate specificity on aldopentose, aldohexose and disaccharides (maltose and cellobiose). But the enzyme did not act on lactose and melibiose.

In our laboratory, physiological properties of alkalophilic bacteria have been studied with the result that the intracellular conditions were not so far from those of the neutral bacteria obtained [19—20]. But the sugar dehydrogenase system of alkalophilic bacteria has not been described. This is the first report on the properties of sugar dehydrogenase of alkalophilic bacteria.

The purified NAD^+-dependent maltose dehydrogenase was composed of a single peptide chain (M_r 40 000 ± 2000) and did not have a subunit structure. This property was unique in comparison with already reported data suggested about dehydrogenase [4,9]. The optimal pH (10.2) of this enzyme is located slightly on the alkaline side compared with sugar dehydrogenase from *Pseudomonas* [3,5,22]. *Gluconobacter* [7] and *Bacillus* [9]. The substrate specificity of our enzyme was different from the enzymes so far reported. Our enzyme could oxidize lactose, maltose, cellobiose and maltotriose in the same degree and also, maltotetraose and maltopentaose were able to become substrates. This enzyme showed the highest affinity on lactose in sugars tested and the K_m value (0.49 mM) was smaller than that of lactose dehydrogenase of *P. graveolens* (11 mM) [17]. The K_m value for maltose, 2.1 mM at pH 10.0, was also smaller than that for maltose of D-galactose dehydrogenase (15 mM) [18]. The primary product from maltose by this enzyme action was estimated as maltono-δ-lactone and it was converted to maltobionic acid. The product contained no keto-group and it was clear that our enzyme was different from the 3-keto-sucrose-forming enzyme of *Agrobacterium tumefaciens* [22] in the catalytic activity on the substrates. The complete inhibition on the enzyme by *p*-chloromercuribenzoic acid, Hg^{2+} and Ag^{2+} suggested that a sulfhydryl group(s) was contributory for the activity.

Acknowledgements

The authors are indebted to Dr. H. Ueyama for his helpful advice and to Drs. N. Nakamura and K. Watanabe for their courtesy in supplying many kinds of chemical. The authors also wish to thank Mr. M. Chijimatsu of the Institute for his help with ultracentrifugation.

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