Alternative Pathways for the Biosynthesis of Alginate from Fructose and Glucose in *Pseudomonas mendocina* and *Azotobacter vinelandii*

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The incorporation of specifically labelled sugars into alginate by mucoid strains of both *Pseudomonas mendocina* and *Azotobacter vinelandii* resulted in substantially different labelling patterns for fructose and glucose. Alginate was synthesized principally from degradation products of glucose, whereas the majority of the polymer produced from fructose was assembled from intact hexose units. A possible explanation for the limited synthesis of alginate from undegraded glucose molecules is that one of the necessary enzymes, glucose-phosphate isomerase, is subject to inhibition by 6-phosphogluconate, a metabolite involved in the catabolism of sugars via the Entner–Doudoroff pathway in both of the organisms.

INTRODUCTION

The pathway of alginic acid biosynthesis in the brown alga *Fucus gardneri* has been described by Lin & Hassid (1966). Early studies suggested that the biosynthesis of bacterial alginate occurred via a similar pathway, since the necessary enzymes were detected in alginate-producing strains of both *Azotobacter vinelandii* (Pindar & Bucke, 1975) and *Pseudomonas aeruginosa* (Piggott *et al.*, 1981). More recently, Banerjee *et al.* (1983) failed to detect phosphomannose isomerase (PMI) and phosphomannomutase, both enzymes of the putative biosynthetic pathway, in alginate-producing strains of *P. aeruginosa*. With the aid of mutants possessing known enzymic deficiencies, these authors demonstrated the involvement of enzymes of the Entner–Doudoroff pathway in the synthesis of alginate from glucose or gluconate by this organism and it was proposed that glyceraldehyde 3-phosphate was a precursor in the biosynthesis of the polymer.

Studies on the incorporation of isotope from specifically labelled glucose (Lynn & Sokatch, 1984) showed that, although there was extensive incorporation of C-6 from glucose into alginate in both *A. vinelandii* and *P. aeruginosa*, only a small proportion of C-1 and C-2 was incorporated into polymer. It was suggested that the synthesis of uronic acid monomers occurred preferentially from C-4, C-5 and C-6 of glucose by the condensation of triose phosphates.

Although PMI was not detected in alginate-producing strains of *P. aeruginosa*, incorporation of a cloned gene specifying PMI activity restored alginate synthesis in an alginate-negative mutant (Darzins *et al.*, 1985); this implies that PMI is essential for alginate synthesis from glucose, despite the fact that the enzyme could not be detected by the normal assay method. Recently, Gill *et al.* (1986) reported that PMI activity could be detected when the gene was overexpressed. A non-mucoid variant of *A. vinelandii* was found (Horan *et al.*, 1983) to lack the key enzymes of alginate biosynthesis, and non-mucoid variants of *P. aeruginosa* have been reported (Pugashetti *et al.*, 1983) to lack GDP-mannose dehydrogenase.

Abbreviations: FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; GDH, glycerol-3-phosphate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; 6PG, 6-phosphogluconate; PGI, glucose-6-phosphate isomerase; PMI, phosphomannose isomerase; TPI, triose-phosphate isomerase.

Fructose-1,6-bisphosphate aldolase has been shown to be an essential enzyme in alginate biosynthesis from glucose or gluconate by *P. aeruginosa* (Banerjee *et al.*, 1985). Thus, the principal pathway of alginate formation from these carbon sources appears to involve degradation via the Entner-Doudoroff pathway, followed by synthesis of fructose 1,6-bisphosphate (FBP) from two molecules of glyceraldehyde 3-phosphate by the (reverse) aldolase reaction; subsequent dephosphorylation to fructose 6-phosphate (F6P) would enable polymer synthesis to continue via the reactions that were originally proposed for the synthesis of bacterial alginate.

Whilst the Entner-Doudoroff pathway is clearly involved in synthesis of alginate from glucose, it is apparent from the data of Banerjee *et al.* (1983, 1985) that washed cell suspensions of mutant strains of *P. aeruginosa* having lesions in glucose-6-phosphate dehydrogenase (G6PD), 2-keto-3-deoxy-6-phosphogluconate aldolase, glucose-6-phosphate isomerase (PGI) or fructose-1,6-bisphosphatase retain the ability to produce alginate from fructose. This finding indicates that the polymer must be synthesized from fructose via an alternative pathway.

We have compared the incorporation of isotope into alginate from specifically labelled fructose and glucose by efficient alginate-producing strains of *P. mendocina* and *A. vinelandii* and suggest an explanation for the differences observed.

METHODS

Growth of P. mendocina in continuous culture. *P. mendocina* NCIB 11687 was grown under nitrogen limitation in a chemostat at a low dissolved oxygen tension (40 ± 5 mmHg); the pH was controlled at 7.0 ± 0.1 and the dilution rate was 0.05 h^{-1} . The medium contained (g l^{-1}): glucose, 50; $(\text{NH}_4)_2\text{HPO}_4$, 1.25; K_2HPO_4 , 1.25; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; citric acid, 0.99; yeast extract (Oxoid), 0.2; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1.1×10^{-3} ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2×10^{-4} ; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 2.8×10^{-4} ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2.5×10^{-4} ; H_3BO_3 , 6×10^{-5} ; and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 3.6×10^{-3} . The glucose was autoclaved separately.

Growth of bacteria in batch culture. *A. vinelandii* SM 52B (Tate and Lyle Ltd) was grown in the medium of Horan *et al.* (1983), except that either fructose or glucose replaced sucrose as the carbon source. *P. mendocina* NCIB 11687 was grown in medium similar to that described by Jayasuria (1955), but with an elevated phosphate concentration (100 mM-total phosphate) to improve the buffering capacity and either fructose or glucose as carbon source. The growth media were autoclaved without the sugars, which were separately sterilized by membrane filtration. For isotopic experiments, flasks (1 l) containing medium (250 ml) were used; the radioisotope [approximately $2 \mu\text{Ci}$ (74 kBq)] was filter-sterilized with the carbon source. Samples of growth media were retained to determine the exact specific activity of the carbon source present in each flask. Bacteria were subcultured twice in the appropriate medium prior to inoculation (1%, v/v) and the flasks were incubated at 30°C , with shaking, until the carbon source was exhausted. In the case of fructose cultures of *P. mendocina*, growth was very slow and it was necessary to add a protease to the growth medium to decrease degradation of the alginate by alginate lyase; Neutrase (Novo; 1 AU l^{-1}) was included in the growth medium.

Preparation of specifically labelled fructose. $[1\text{-}^{14}\text{C}]$ Fructose and $[6\text{-}^{14}\text{C}]$ fructose were prepared from specifically labelled glucose with the aid of immobilized glucose isomerase. Glucose (0.5 g) and specifically labelled glucose [$20 \mu\text{Ci}$, 55 Ci mol^{-1} (340 kBq, 2.03 TBq mol^{-1})] were incubated with Sweetzyme Q (Novo; 0.1 g) in a total volume of 1 ml. The reaction was allowed to proceed until equilibrium was attained (50 h), at which time fructose and glucose were present in approximately equal concentrations. The sugar solution was removed and heated at 100°C for 10 min to inactivate any solubilized enzyme.

To facilitate the separation of fructose from the mixture of sugars, the glucose was selectively phosphorylated. The mixture was incubated at 37°C in 50 mM-sodium phosphate buffer (50 ml, pH 7.0) containing glucokinase (2.5 U), MgCl_2 (5 mM) and ATP (1 g) until no free glucose remained. The solution was passed through a column of Dowex 1×2 (carbonate form) resin, followed by Dowex 50W $\times 8$ (ammonium form) resin to remove glucose 6-phosphate and buffer. Ammonium carbonate was removed by repeated rotary evaporation at 30°C . The specifically labelled fructose was shown to be free of phosphates and the extent of contamination by glucose, resulting from the non-enzymic interconversion of the sugars in alkaline solution, was less than 2% (w/w).

Analytical methods. Glucose was estimated by the glucose oxidase method, using a commercial kit (Boehringer). Fructose was determined by the resorcinol method (Herbert *et al.*, 1971). The method of Kalb & Bernlohr (1977) was used to determine the concentration of protein in cell-free extracts. Alginate was determined both by uronic acid analysis (Blumenkrantz & Absoe-Hansen, 1973), using commercial alginic acid (Sigma, type III) as standard, and gravimetrically by drying samples to constant weight at 110°C . The radioactivity of aqueous solutions was measured by liquid scintillation counting in the scintillant of Bray (1960).

Isolation and purification of alginate. Radiolabelled cultures were centrifuged (4°C) at $40000 g$ for 30 min to sediment the bacteria and the supernatant solution was centrifuged again, under the same conditions, to ensure

that no bacteria remained in the crude alginate solution. In the case of *A. vinelandii* cultures, NaCl (100 mM) and EDTA (disodium salt, 10 mM) were incorporated, prior to centrifugation, to facilitate clarification of the alginate solution, as described by Horan *et al.* (1983).

Alginate was precipitated from the supernatant solution (200 ml) by shaking with 3 vols propan-2-ol; the gel so obtained was dissolved in the minimum volume of water (100–300 ml) by vigorous stirring and reprecipitated twice from solution. The alginate solution was subjected to exhaustive dialysis (4 °C) against water to remove traces of propan-2-ol and low- M_r material. The specific activity of the resulting viscous alginate solution was determined.

To measure the yield of alginate produced by *A. vinelandii*, cultures were harvested after exhaustion of the carbon source and the polymer was recovered from measured volumes of supernatant fluid by a single precipitation with propan-2-ol; it was established that no more alginate could be precipitated by increasing the concentration of propan-2-ol.

Enzyme assays. All assays were carried out at 30 °C. Cell-free extracts were prepared essentially as described by Mitchell & Dawes (1982).

Fructokinase (EC 2.7.1.4) and 1-phosphofructokinase (EC 2.7.1.56) were assayed by the methods of van Dijken & Quayle (1977).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed as described by Anderson & Dawes (1985), except that the pH of the buffer was 8.3.

Glucose-6-phosphate isomerase (EC 5.3.1.9) was assayed in a mixture (1 ml) containing Tris/HCl buffer (pH 8.2 at 30 °C, 50 μ mol), DTT (5 μ mol), NADP (1 μ mol), F6P (4 μ mol) and G6PD (5 U). The reaction was initiated by addition of the cell extract after contaminating glucose 6-phosphate, present in the F6P, had been consumed. For inhibition studies with 6-phosphogluconate (6PG), NaF (50 mM) was included in the assay to inhibit 6-phosphogluconate dehydratase (Kovachevich & Wood, 1955).

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) was assayed in a mixture (1 ml) containing sodium borate buffer (pH 9.3, 50 μ mol), DTT (1 μ mol), MgSO_4 (6.3 μ mol), FBP (1 μ mol), NADH (0.15 μ mol), KCl (63 μ mol), triose-phosphate isomerase [TPI (EC 5.3.1.1): 26 U] and glycerol-3-phosphate dehydrogenase [GDH (EC 1.1.99.5): 4 U].

Fructose-1,6-bisphosphatase (EC 3.1.3.11) was assayed in a mixture (1 ml) containing sodium borate buffer (pH 9.5, 50 μ mol), MgCl_2 (20 μ mol), NADP (3 μ mol), FBP (2 μ mol), PGI (5 U) and G6PD (2 U).

Phosphofructokinase (EC 2.7.1.11) was assayed in a mixture (1 ml) containing Tris/HCl buffer (pH 8.0 at 30 °C, 40 μ mol), DTT (1 μ mol), MgSO_4 (6.3 μ mol), KCl (63 μ mol), ATP (3.6 μ mol), NADH (0.3 μ mol), TPI (11 U) and GDH (2 U).

Other enzyme assays were carried out by the procedures described, or referred to, by Mitchell & Dawes (1982). Specific activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

Materials. Specifically labelled glucose [50–60 mCi mmol^{-1} (1.85–2.22 GBq mmol^{-1})] was obtained from Amersham.

Glucokinase was purified by affinity chromatography (Doelle, 1982) from *Zymomonas mobilis* CP4. The organism was grown and cell-free extracts were prepared as described by Anderson & Dawes (1985). The purified enzyme possessed no fructokinase activity.

Other enzymes and biochemicals were purchased from Sigma.

RESULTS AND DISCUSSION

Incorporation of specifically labelled sugars into alginate

The organisms chosen for this study are mutants of *A. vinelandii* and *P. mendocina* that have previously been selected for their ability to produce enhanced concentrations of alginic acid (Horan *et al.*, 1983; Hacking *et al.*, 1983). Fructose supported growth and alginate production in both organisms, although the rate of uptake was lower than that of glucose (Fig. 1), especially in the case of *P. mendocina*. The related organism, *P. aeruginosa*, has also been reported to exhibit a low growth rate with fructose as the sole carbon source (Sawyer *et al.*, 1977).

The initial concentration of fructose and glucose for the radioactive-labelling experiments was chosen, in each case, as the highest concentration that was completely utilized in batch culture, without being preceded by a period of very slow uptake. The utilization of fructose and glucose by both organisms is shown in Fig. 1. In the case of *P. mendocina*, fructose utilization was slow and incomplete, even at low substrate concentration (10 g l^{-1}), and recovery of alginate from the culture by precipitation was very poor because the polymer was extensively degraded by alginate lyase during the extended incubation period. The inclusion of a proteolytic enzyme

Fig. 1

Fig. 2

Fig. 1. Utilization of fructose (open symbols) and glucose (filled symbols) by *A. vinelandii* (Δ, ▲) and *P. mendocina* (○, ●) growing in shake-flask cultures at 30 °C.

Fig. 2. Inhibition of G6PD from *P. mendocina* by ATP, with NAD (1 mM) as cofactor. A constant amount of enzyme (0.15 mg protein) was present in each assay. ATP concentrations (mM): ●, 0; Δ, 1; ○, 2.5.

Table 1. Incorporation of specifically labelled glucose and fructose into alginate

Organism	Substrate	Relative specific activity of alginate*	Standard deviation	No. of expts
<i>A. vinelandii</i>	[1- ¹⁴ C]Glucose	0.13	0.03	2
	[2- ¹⁴ C]Glucose	0.30	0.01	2
	[6- ¹⁴ C]Glucose	1.76	0.10	2
<i>P. mendocina</i>	[1- ¹⁴ C]Glucose	0.08	0.01	3
	[2- ¹⁴ C]Glucose	0.35	0	2
	[6- ¹⁴ C]Glucose	1.54	0.02	3
<i>A. vinelandii</i>	[1- ¹⁴ C]Fructose	0.78	0.10	4
	[6- ¹⁴ C]Fructose	1.08	0.03	3
<i>P. mendocina</i>	[1- ¹⁴ C]Fructose	0.91	0.13	3
	[6- ¹⁴ C]Fructose	1.11	0.09	4

* These values represent the specific activity of purified alginate, relative to that of the growth substrate. The relative specific activities are calculated as molar ratios. These calculations assume a value of 176 for the effective M_r of the uronic acid repeating unit in the polymer.

in the culture medium to inactivate the alginate lyase, as described by Hacking *et al.* (1983), allowed an improved recovery of alginate.

The purification of alginate from the radiolabelled cultures involved repeated precipitation of the polymer, followed by dialysis. This procedure was carried out to remove labelled material entrapped in the alginate gel during precipitation.

The incorporation of specifically labelled glucose into alginate by *A. vinelandii* and *P. mendocina* is summarized in Table 1. The results show that, whereas only a small proportion of C-1 or C-2 of glucose was incorporated into alginate by either organism, the specific activity of alginate synthesized from [6-¹⁴C]glucose was substantially higher than that of the substrate, in both cases. These results are in good agreement with those of Lynn & Sokatch (1984) for the incorporation of labelled glucose into alginate by *A. vinelandii* and *P. aeruginosa* and confirm that the principal pathway of alginate biosynthesis involves cleavage of the glucose molecule, presumably via the Entner–Doudoroff pathway, as proposed by Banerjee *et al.* (1983, 1985). The small, but significant, amount of radioisotope recovered in alginate from bacteria grown on 1- or 2-labelled glucose probably represents synthesis of polymer from intact hexose units.

Table 2. Comparison of the activities of the enzymes involved in the utilization of fructose by *P. mendocina* and *A. vinelandii*

The values presented are means of the activities of the enzymes in two cell-free extracts prepared from bacteria grown in batch culture with fructose as sole carbon source.

Enzyme	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	<i>A. vinelandii</i>	<i>P. mendocina</i>
Fructokinase	220	ND
1-Phosphofructokinase	ND	230
Fructose-1,6-bisphosphatase	20	22

ND, Not detected.

In contrast to the results obtained with specifically labelled glucose, there was substantial incorporation of both 1- and 6-labelled fructose into alginate by both organisms (Table 1). The greater incorporation of 6-labelled glucose again suggests the operation of parallel pathways, but in the case of fructose, the synthesis of polymer via the Entner–Doudoroff pathway is of minor importance, compared with direct synthesis from undegraded hexose units.

Pathways of fructose and glucose metabolism

Although similar results were obtained for the incorporation of specifically labelled glucose and fructose into both *A. vinelandii* and *P. mendocina*, the mode of fructose uptake differed in these organisms. *A. vinelandii* was found to have an ATP-dependent fructokinase or hexokinase (Table 2), whereas *P. mendocina* possessed a 1-phosphofructokinase, in common with other pseudomonads, in which fructose is utilized via a fructose phosphotransferase system (Lessie & Phibbs, 1984). Fructose-1,6-bisphosphatase activity was detected in cell-free extracts of both organisms (Table 2).

In an attempt to provide an explanation for the preferred indirect route of alginate synthesis from glucose, we investigated the metabolism of this substrate in *P. mendocina*. The organism was grown in continuous culture, under nitrogen limitation, whereby the yield of alginate is substantially greater than the biomass concentration (Hacking *et al.*, 1983). The specific activities, in crude extracts, of various enzymes involved in the metabolism of glucose are given in Table 3.

Glucose 6-phosphate can be converted directly to F6P, the apparent precursor of alginate synthesis, by the action of PGI, or may be channelled through the Entner–Doudoroff pathway by the action of G6PD. The activity of PGI was substantially less than that of G6PD (Table 3). G6PD was found to be a regulatory enzyme, active with both NAD and NADP, for which ATP is a negative allosteric effector (Fig. 2). Although PGI could readily be assayed in the 'reverse' direction in a coupled assay using F6P as substrate, no satisfactory assay could be developed for the enzyme in the 'forward' direction, to allow the K_m value for G6P to be evaluated, but a value of 0.29 mM was determined with F6P as substrate. 6PG is a competitive inhibitor of PGI with F6P as substrate, and a K_i value of 1.2 mM was calculated for this inhibitor. The inclusion of NaF (50 mM) to inhibit 6PG dehydratase blocked this reaction at the maximum concentration (5 mM) of 6PG employed in the inhibition studies, indicating that the true inhibitor was 6PG, rather than some metabolite produced from this compound. Competitive inhibition of PGI by 6PG has previously been found with enzyme derived from peas (Hizukuri *et al.*, 1975), cardiac tissue (Gracy & Tilley, 1975) and *Bacillus stearothermophilus* (Nosoh, 1975); K_i values for 6PG of 13 μ M and 63 μ M have been reported for the pea and cardiac enzymes, respectively, in assays with G6P as substrate.

It is likely that during growth of *P. mendocina* in medium containing glucose, the intracellular concentration of 6PG is sufficient to restrict the isomerization of G6P, so that the principal route of alginate synthesis is via the dehydrogenase rather than via the isomerase. Competitive inhibition of PGI by 6PG was also demonstrated in *A. vinelandii* (results not shown).

Fig. 3. Alternative pathways leading to algininate biosynthesis. — *P. mendocina* and *A. vinelandii*; *P. mendocina*; ---- *A. vinelandii*. Abbreviations: F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; Pyr, pyruvate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate.

Table 3. Activities of various enzymes of glucose catabolism in *P. mendocina*

The values presented are from three algininate-producing continuous cultures (nitrogen-limited) and show the range of activities present in cell-free extracts.

Enzyme	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]
Glucokinase	83–164
Glucose dehydrogenase	127–159
Gluconate dehydrogenase	ND
Gluconate kinase	5–8
Glucose-6-phosphate dehydrogenase	274–456
Glucose-6-phosphate isomerase	43–68
Entner–Doudoroff enzymes*	18–26
2-Oxogluconate enzymes†	ND
Fructose-1,6-bisphosphate aldolase	27–39
Fructose-1,6-bisphosphatase	20–30
6-Phosphofructokinase	ND

ND, Not detected.

* Combined assay of 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14).

† Combined assay of dehydroglucokinase (EC 2.7.1.13) and phosphogluconate dehydrogenase (EC 1.1.1.43).

The oxidation of glucose to 2-ketogluconate, via gluconate, by membrane-bound dehydrogenases prior to transport across the cytoplasmic membrane is an important route of glucose metabolism in *P. aeruginosa* (Midgley & Dawes, 1973; Roberts *et al.*, 1973). Although glucose dehydrogenase was present in *P. mendocina*, neither gluconate dehydrogenase nor 2-oxogluconate kinase/reductase activities were detected. In addition, washed cell suspensions of *P. mendocina* failed to produce 2-ketogluconate and this compound was not a substrate for the growth of the organism; *P. aeruginosa* was used as a positive control for these experiments (results not shown). Gluconate was detected in the culture medium when *P. mendocina* was grown under nitrogen limitation in glucose medium, but only at dilution rates lower than 0.05 h⁻¹ (Sengha, 1985).

Pseudomonas strains normally lack 6-phosphofructokinase and are unable to degrade sugars via the Embden–Meyerhof pathway. Banerjee (1986) has recently provided evidence for the existence of this enzyme in mutant strains of mucoid *P. aeruginosa* but the significance of this

finding, in relation to alginate biosynthesis, has yet to be demonstrated. 6-Phosphofructokinase was not detected in *P. mendocina*.

The apparent pathways available for glucose and fructose metabolism in *P. mendocina* and *A. vinelandii* are summarized in Fig. 3.

The biosynthesis of alginate from triose phosphates is less efficient than direct synthesis from hexose monomers since the dephosphorylation of FBP effectively wastes ATP; this suggests that, at least in *A. vinelandii*, fructose might be a better substrate than glucose for efficient conversion to alginate. This was found to be the case for batch cultures of *A. vinelandii*. Comparisons were made of the yield of alginate and biomass in batch culture of *A. vinelandii* grown under identical conditions, with glucose or fructose (15 g l^{-1}) as the carbon source. In six experiments with each substrate, glucose yielded $[g (\text{mol substrate})^{-1}]$ 37.8 ± 3.2 alginate and 20.8 ± 3.2 biomass, while fructose yielded 45.2 ± 4.6 alginate and 15.0 ± 2.7 biomass. Comparison of the alginate yields was not attempted for *P. mendocina* because the polymer is degraded, to some extent during growth, by an alginate lyase and quantitative recovery is difficult. In this organism, utilization of both substrates by their preferred routes involves the dephosphorylation of FBP.

Since alginate synthesized from glucose via the degradative route contains only three of the six carbon atoms present in the substrate, the conversion efficiency is limited to approximately 50%. This conversion efficiency has been realized for *P. mendocina* U1 under optimum growth conditions in continuous culture (Sengha, 1985).

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