

Preparation of D-xylulose from D-xylose

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A simple method is described for the preparation of D-xylulose. It consists of the isomerization of D-xylose with xylose isomerase (EC 5.3.1.5), yielding an equilibrium mixture of D-xylulose and D-xylose. This is followed by the quantitative oxidation of residual D-xylose to D-xylonic acid with immobilized A. calcoaceticus cells. A combination of methanol precipitation and ion exchange is used for the removal of xylonic acid. This procedure offers many advantages over existing methods for the preparation of D-xylulose. The purity of the final product compares favorably to that of a commercial D-xylulose preparation.

Keywords: D-xylulose; xylose isomerase; *Acinetobacter calcoaceticus*; immobilization

Introduction

D-Xylulose is an important intermediate in the alcoholic fermentation of D-xylose by yeasts.^{1,2} Because of the potential economic significance of this process, much attention has been paid to the mechanism and regulation of xylose fermentation (for a review see reference 3). Investigations into xylose fermentation and other metabolic pathways involving D-xylulose or derivatives as intermediates may depend on the availability of D-xylulose as a model substrate.

In the literature a number of methods have been described for the production of D-xylulose. Chemical methods^{4,5} generally give low yields, and the formation of isomers is difficult to avoid. Biological methods using either arabinitol or xylose as a starting material are more specific and have been reported to give higher yields.⁶⁻⁸ Conversions of 40 to 60% were reported^{6,7} for the oxidation of arabinitol to D-xylulose by *Acetobacter suboxydans* (now called *Gluconobacter oxydans*) and mutants of *Klebsiella pneumoniae*. In the latter case, separation of D-xylulose from the substrate D-arabinitol was achieved by hydroxyl affinity chromatography.⁷

Partial conversion of D-xylose into D-xylulose can be achieved in cell-free systems using xylose isomerase. This enzyme (EC 5.3.1.5) catalyses the isomeriza-

tion of D-xylose to D-xylulose. However, separation of D-xylose and D-xylulose is difficult with standard physical techniques. Therefore, microorganisms exhibiting sequential use of D-xylose and D-xylulose have been applied for the removal of D-xylose from isomerization mixtures.⁸

An interesting alternative to procedures reported previously is the use of bacteria capable of oxidizing D-xylose to D-xylonic acid. For example, *Acinetobacter calcoaceticus* is unable to grow on D-xylose or D-xylulose,⁹ but quantitatively oxidizes xylose and a number of other aldose sugars to the corresponding aldonic acids. This reaction is catalysed by a membrane-bound glucose dehydrogenase (EC 1.1.99.17).¹⁰

In this paper, a method for the production of D-xylulose is described, which is based on the elimination of D-xylose by *A. calcoaceticus* (Figure 1). For the removal of D-xylonic acid, a combination of methanol precipitation and ion exchange was applied.

Material and methods

Organism and growth conditions

Acinetobacter calcoaceticus LMD 79.41 was obtained from the culture collection of the Department of Microbiology and Enzymology, Delft, The Netherlands. The organism was grown in a chemostat under acetate limitation, at a dilution rate of 0.15 h⁻¹ and at 30°C. An Applikon laboratory fermenter with a working volume

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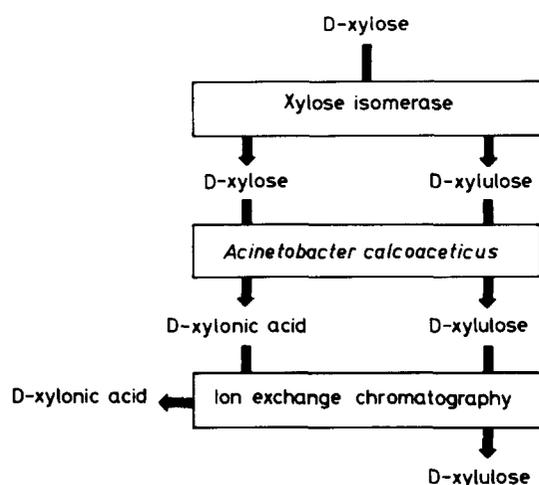


Figure 1 Outline of the procedure used for the preparation of D-xylulose from D-xylose

of 1 l was used. The dissolved oxygen tension was recorded with a steam-sterilizable Clark-type oxygen electrode and was kept constant at 40% of air saturation. In order to prevent foaming, air was blown over, rather than sparged through the culture. The pH was controlled at 7.0 by automatic addition of 0.5 M H_2SO_4 . The mineral medium was prepared according to van Schie *et al.*¹¹ It contained 30 mM sodium acetate.

Immobilization of *A. calcoaceticus*

A cell suspension (30 l) obtained from acetate-limited chemostat cultures was centrifuged at 20 000g using a continuous rotor. The resulting cell pellet was resuspended in 5 l potassium phosphate buffer (50 mM; pH 7.0) to a final density of 2.0 g dry weight l^{-1} . Sodium alginate was added under vigorous stirring to a final concentration of 2% (w/v). Droplets were generated with a resonance nozzle¹² and stabilized in a stirred 0.1 M $CaCl_2$ solution. The average diameter of the biocatalyst beads was 1.0 mm.

Isomerization of D-xylose to D-xylulose

Immobilized xylose isomerase (EC 5.3.1.5) was obtained from Gist-brocades, Delft, The Netherlands (Maxazyme GI IMMOB). The beads were washed four times with demineralized water before use. Isomerization was carried out at 70°C in a thermostated vessel containing 1 l 30 mM $MgCl_2$ and 3.3 M D-xylose. The reaction was initiated by the addition of 250 g of immobilized xylose isomerase. During isomerization the mixture was gently stirred. The pH of the mixture was maintained at 7.0 by periodic addition of 0.1 M NaOH. D-Xylose and D-xylulose concentrations were measured at regular intervals using high-performance liquid chromatography (HPLC) (see below). After equilibrium had been reached, the resulting xylose-xylulose solution was decanted and stored at -20°C.

Oxidation of D-xylose

The oxidation step was carried out at 35°C in an airlift reactor with a working volume of 10 l, containing 7.5 l 0.5% (w/v) $CaCl_2$ and 0.5 l of the isomerization mixture, yielding initial xylose and xylulose concentrations of 160 and 60 mM, respectively. The amount of biomass in the reactor amounted to 10 g dry weight of cells. The reaction mixture was aerated with a mixture of air and pure oxygen. The dissolved oxygen concentration was maintained at 400 μM by adjustment of the relative amounts of air and oxygen in the inlet gas. The pH of the reaction mixture was maintained at 6.5 by the automatic addition of 4 M NaOH. Acid formation was calculated from the weight of the NaOH solution added to the reactor. At the end of the process, the pH of the reaction mixture was increased to 8.2 in order to hydrolyse any residual xylonolactone.

Purification of D-xylulose

After the oxidation step, the biocatalyst beads were removed by filtration over cheesecloth. The resulting solution (6 l) was filtered over a 0.20- μm pore size capsule filter (Acroflux, Gelman Sciences Inc., Ann Arbor, Michigan, USA) and concentrated to 400 ml with a rotating vacuum evaporator at 50°C. To the concentrate, 800 ml of methanol was added dropwise over a period of 1 h, at 50°C. The precipitate was redissolved in 250 ml of demineralized water and again precipitated. Methanol was removed from the combined soluble fractions by vacuum evaporation. A precipitate consisting mainly of inorganic salts formed during vacuum evaporation; it was removed by filtration. Propylene glycol was removed from the resulting 250 ml D-xylulose-rich solution by three consecutive extractions with 250 ml of *n*-butanol. D-Xylonic acid and inorganic salts still present after the methanol precipitation step were removed by ion exchange. Two hundred fifty milliliters of D-xylulose-rich solution was diluted with 1 l of demineralized water, after which Dowex MSC-1 (H^+) and Dowex 11 (OH^-) resins were added (500 g each). Ion exchange was carried out for 30 min at 0°C in a stirred tank. During ion exchange, the pH was monitored continuously and was found to remain slightly acidic (pH 4–6). The D-xylulose solution obtained after filtration was lyophilized and stored at -20°C.

Carbohydrate determinations

D-Xylose and D-xylulose were measured with a Waters M6000-A HPLC system, equipped with a Bio-Rad HPX 87-C carbohydrate column and a Waters R401 refractive index detector. Data were processed with a Spectra Physics SP4270 integrator unit. A 10 mM calcium phosphate solution (pH 2.0) (F. van Rantwijk, personal communication) was used as the eluent. Under these conditions, D-xylulose and xylonolactone peaks overlapped. Accurate determination of D-xylulose in samples taken during the oxidation step was only possible after hydrolysis of xylonolactone to

xylonic acid. To this end, the pH of the samples was raised to 8.5. After 10 min incubation at room temperature, the samples were neutralized and injected. Before analysis, samples were diluted with demineralized water to give final sugar concentrations of 0.2 to 2 g l⁻¹. The sample volume was set at 100 μ l (Perkin-Elmer ISS-100 autosampler). D-Xylonic acid was measured on the same column. Xylonic acid standards were prepared as described previously.¹³

Measurements of xylose-dependent oxygen consumption

D-Xylose oxidation by suspensions of intact cells or immobilized cells was assayed by following the rate of oxygen consumption with a Clark-type oxygen electrode in a reaction volume of 4 ml at 35°C. Cells from acetate-limited chemostat cultures were harvested by centrifugation and resuspended in 50 mM potassium phosphate buffer (pH 6.5). Oxygen uptake by alginate-immobilized *A. calcoaceticus* cells was assayed in 0.5% (w/v) CaCl₂ adjusted to pH 6.5.

Total organic carbon measurements

A Beckman model 915 B Tocamaster total organic carbon analyzer was used to determine the carbon content of cell suspensions and culture supernatants, the carbon content of the bacteria being obtained from the difference. Bacterial dry weight was calculated assuming a carbon content of 50%.

Chemicals

D-Xylose was obtained from Merck (Darmstadt, FRG). D-Xylulose was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium alginate was from Fluka AG (Buchs, Switzerland).

Results

Isomerization of D-xylose to D-xylulose

A mixture of D-xylose and D-xylulose was prepared with immobilized xylose isomerase. Under the experimental conditions used, equilibrium between D-xylose and D-xylulose was reached after approximately 1.5 h. The equilibrium mixture consisted of approximately 27% D-xylulose and 73% D-xylose (Figure 2). Apart from D-xylose and D-xylulose, two other compounds were detectable in the isomerization mixture (Figure 3A). Propylene glycol originated from the immobilized xylose isomerase, in which it was present as a preservative. Contamination with propylene glycol can be avoided by extensive washing of the enzyme before use (data not shown). Alternatively, propylene glycol can be removed by repeated extractions with *n*-butanol. A second contaminating compound (Figure 3A) was identified as D-ribulose. The retention time and the ratio of u.v.-detector (215 nm) and r.i.-detector signals were identical to those of a sample of D-ribulose. D-Ribulose is formed upon base-catalysed epimerization at the C-3 atom of d-xylulose.¹⁴ Suppres-

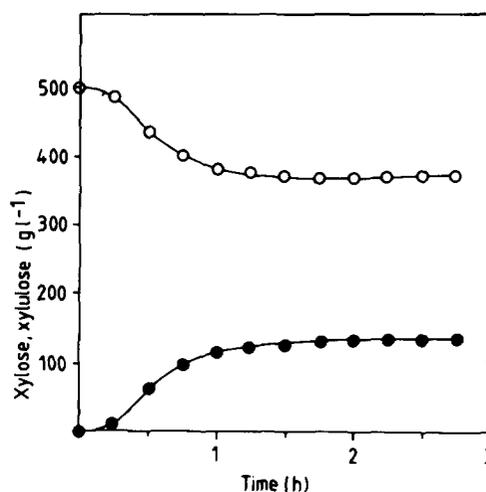


Figure 2 Isomerization of D-xylose to D-xylulose with immobilized xylose isomerase. Isomerization was carried out at 70°C and at pH 7.0. Immobilized xylose isomerase was present in a concentration of approximately 1500 units ml⁻¹. ○, D-xylose; ●, D-xylulose

sion of this side reaction by lowering of the isomerization temperature, pH, or reaction time (e.g. by using xylose isomerase from a different source) is under investigation. D-Ribulose amounted to approximately 2% of the total amount of D-xylulose present in the isomerization mixture.

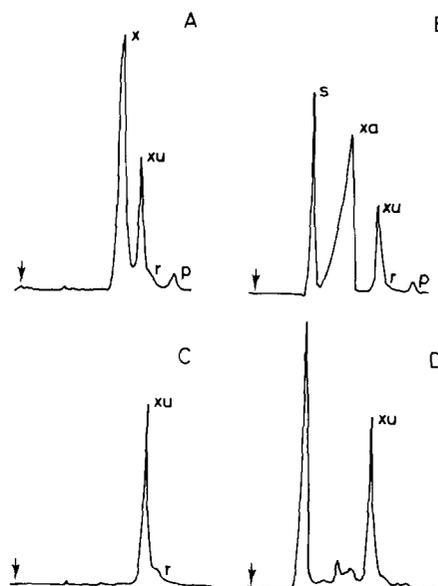


Figure 3 HPLC profiles of samples taken at different stages of the D-xylulose production process and from a commercial D-xylulose preparation. (A) Mixture obtained after isomerization of D-xylose with immobilized xylose isomerase. (B) Mixture obtained after oxidation of D-xylose with immobilized cells of *A. calcoaceticus*. (C) Final D-xylulose preparation. (D) Commercial D-xylulose preparation. Injection time is indicated by arrows. Abbreviations indicate the following compounds: p, propylene glycol; r, ribulose; s, inorganic salts; x, xylose; xa, xylonic acid; xu xylulose

Table 1 Oxidation of D-xylose by cell suspensions and immobilized cells of *A. calcoaceticus* LMD 79.41

D-Xylose concentration	Rate of xylose oxidation	
	Cell suspensions	Immobilized cells
20 mM	340	160
500 mM	260	n.d.

The rate of oxygen consumption was recorded at 35°C and corrected for endogenous respiration. Activities are expressed as $\text{nmol O}_2 \text{ min}^{-1} (\text{mg dry weight})^{-1}$
n.d., Not determined

Immobilization of *A. calcoaceticus*

Immobilization of *A. calcoaceticus* cells led to a decrease in their xylose-oxidizing activity (Table 1). Furthermore, xylose-oxidizing activity of cell suspensions was reduced at high xylose concentrations (Table 1). Substrate inhibition of glucose dehydrogenase from *A. calcoaceticus* has been described previously.¹⁵

In contrast to intact cells, of which the oxidation rates were constant over a wide range of oxygen concentrations (data not shown), immobilized cells showed rates of xylose oxidation that were strongly dependent upon the oxygen concentration (Figure 4). At dissolved oxygen concentrations lower than 400 μM , the rate of xylose oxidation was limited by oxygen diffusion. It should be stressed that the occurrence of oxygen diffusion limitation may strongly depend upon the size of the alginate beads and the ratio of cells versus alginate used for immobilization.¹⁶ No attempts have been made to optimize either of these two parameters.

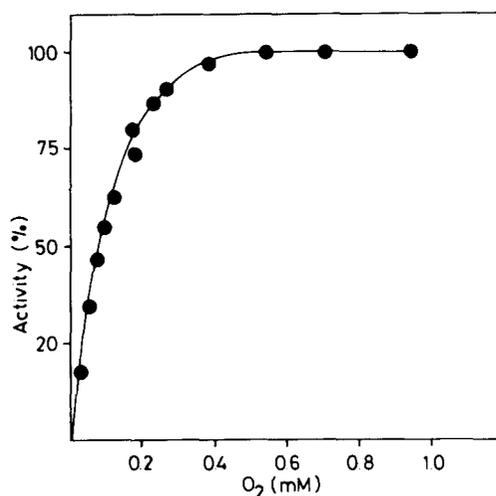


Figure 4 Effect of oxygen concentration on D-xylose oxidation by calcium alginate-entrapped *A. calcoaceticus* cells. One gram wet weight of biocatalyst beads was added to 4 ml of 0.5% (w/v) CaCl_2 (pH 6.5). Oxygen uptake was assayed at 35°C with a Clark-type oxygen electrode. 100% activity corresponds with a D-xylose-oxidizing activity of $320 \text{ nmol min}^{-1} (\text{mg dry weight of cells})^{-1}$

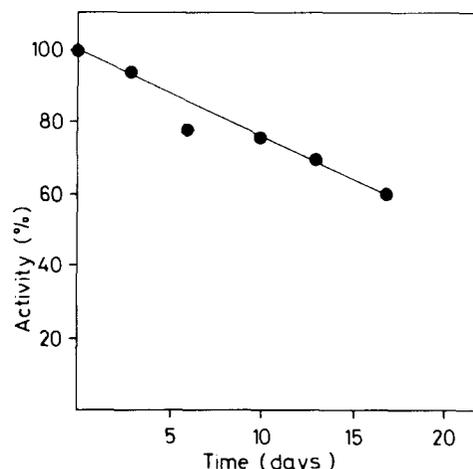


Figure 5 Stability of D-xylose-oxidizing activity of *A. calcoaceticus* cells immobilized in calcium alginate. Biocatalyst beads were stored at 4°C in a 0.5% (w/v) CaCl_2 solution (pH 6.5). D-Xylose-oxidizing activity was assayed at 35°C with a Clark-type electrode. 100% activity corresponds with a D-xylose-oxidizing activity of $320 \text{ nmol min}^{-1} (\text{mg dry weight of cells})^{-1}$

When stored at 4°C in a 0.5% (w/v) solution of calcium chloride, the activity of the immobilized cells decreased linearly with time. In 20 days the original activity was reduced by 50% (Figure 5).

Oxidation of D-xylose by immobilized *A. calcoaceticus* cells

On the basis of the experiments described above, it was decided to perform the oxidation process at an oxygen concentration of 400 μM . The initial D-xylose concentration was 160 mM.

D-Xylose was quantitatively converted into xylonic acid. Under the experimental conditions used, the oxidation was complete after approximately 11 h (Figure 6). The rate of xylose disappearance was higher than the rate of acid production. This observation must be explained by the fact that not xylonic acid, but xylonolactone, is the initial product of xylose oxidation by *A. calcoaceticus*. Lactone hydrolysis is a pH-dependent, nonenzymic process, which may become the rate-limiting step in xylonic acid production at pH values below 7.0.¹³

During the oxidation process, the D-xylulose concentration did not change significantly. This is in agreement with the fact that *A. calcoaceticus* is unable to grow on D-xylulose.⁹ An HPLC profile of the mixture obtained after the oxidation of xylose is shown in Figure 3B.

The airlift reactor and the solutions of CaCl_2 and NaOH used in the oxidation process were heat-sterilized before use. However, neither the isomerization mixture nor the biocatalyst beads were produced under sterile conditions. Therefore, the reaction mixture was regularly checked for infections by phase contrast microscopy. Small numbers of *Pseudomonas*-like rods could be detected only during the last 3 h. The pres-

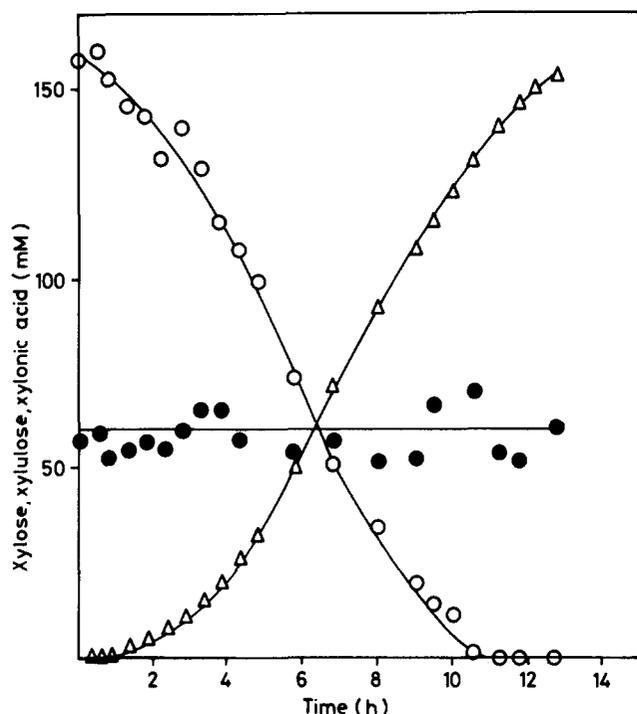


Figure 6 Oxidation of D-xylose by immobilized cells of *A. calcoaceticus*. At $t = 0$, a mixture of D-xylose and D-xylulose obtained with xylose isomerase was added to the reactor. The oxidation was carried out at 35°C and at pH 6.5. Note that after 10.5 h, xylose oxidation is completed, whereas D-xylonic acid production continues. This is due to the chemical hydrolysis of D-xylonolactone, the primary product of D-xylose oxidation. ○, D-xylose; ●, D-xylulose; △, D-xylonic acid

ence of these bacteria did not seem to influence the oxidation reaction, nor did any detectable consumption of xylulose occur at this stage.

Purification of D-xylulose

For the removal of xylonic acid from the oxidation mixture, ion exchange preceded by methanol precipitation was used. The yield of the process was rather low. From an initial 70 g of D-xylulose present in the oxidation mixture, only 10 g of pure D-xylulose was recovered in the final preparation. So far, no attempts have been made to optimize this part of the process.

D-Xylulose was obtained in the form of a clear colorless syrup. Attempts to crystallize xylulose from this syrup have failed so far. Propylene glycol was effectively removed by repeated extractions with *n*-butanol. The only significant contamination present in the final D-xylulose preparation is D-ribulose, which was already present after isomerization. This conclusion was confirmed by ^{13}C -NMR (nuclear magnetic resonance) analysis of the final preparation (data not shown). An HPLC profile of the final D-xylulose preparation is shown in Figure 3C.

Discussion

The aim of the present investigation was to develop a simple procedure for the preparation of pure D-xylu-

lose from D-xylose. This required the quantitative separation of D-xylose from D-xylulose. These sugars are difficult to separate by standard physical methods. Therefore, a method was devised based on the conversion of xylose into a product that can easily be removed. An existing method⁸ relies on the conversion of xylose into carbon dioxide and biomass by selected microorganisms. A major disadvantage of this method is the fact that, although xylose is consumed preferably in a mixture of xylose and xylulose, the metabolism of these sugars is not strictly separated in time. This therefore requires the measurement of sugar concentrations at regular time intervals. In the process outlined in Figure 1 and discussed above, no consumption of D-xylulose occurs during or after xylose oxidation. The process can easily be monitored by following the rate of NaOH titration. Alternatively, the consumption of oxygen can be monitored with a paramagnetic gas analyzer. Furthermore, the increase in the dissolved oxygen concentration at the end of the process also proved to be a reliable indication for the completion of sugar oxidation (results not shown).

Our method relies on the use of ion exchange for the removal of contaminating D-xylonic acid and inorganic salts. This, however, cannot be regarded as a disadvantage, since ion exchange is also essential for the removal of inorganic salts and lysis products from growth media used for the production of D-xylulose via other procedures.

So far, no data are available on the purity of D-xylulose produced by microbiological methods. The commercial preparation (Figure 3D), which is available as a light yellow syrup, contained many impurities, in contrast to our D-xylulose preparation (Figure 3C), which was colorless.

Not only xylulose itself, but also its phosphate derivative xylulose 5-phosphate, is a useful compound in metabolic studies. It is a key intermediate in various pathways of sugar metabolism and methanol assimilation in yeasts.¹⁷ Our D-xylulose preparation seems sufficiently pure to allow preparation of D-xylulose 5-phosphate via phosphorylation of D-xylulose with xylulokinase (EC 2.7.1.17).

Immobilization of *A. calcoaceticus* in calcium alginate prevents the heavy foaming that occurs as a result of vigorous aeration of cell suspensions of this organism and facilitates separation of the product from the cells. The latter advantage is only of minor importance, since free cells can easily be removed by filtration. Apart from *A. calcoaceticus*, some other Gram-negative bacteria are known to oxidize D-xylose to xylonic acid.^{13,18} Application of, for instance, *Gluconobacter oxydans* or *Pseudomonas fragi* might reduce foaming problems and thus circumvent the necessity of immobilizing the cells. The shelf life of the immobilized *A. calcoaceticus* cells would seem to permit reuse of the biocatalyst beads. When reuse is desired, measures should be taken to prevent contamination, for example by the inclusion of antibiotics.

A recent study¹⁸ has shown that xylonic acid may have economically interesting properties. It may

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therefore be advantageous to also purify xylonic acid from the oxidation mixture.

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