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Polyuronic Acids Produced by *Pseudomonas aeruginosa**

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ABSTRACT: Polysaccharides produced by thirteen "mucoid-type" *Pseudomonas aeruginosa* organisms were compared and all were found to be polyuronic acids. The principal component of each polyuronide preparation was D-mannuronic acid, with variable amounts of L-guluronic acid detectable. The polyuronides were partially reduced to the corresponding hexosans by esterification and reduction of the methyl esters with sodium borohydride.

The uronic acids and their corresponding hexoses were identified by paper chromatography and electrophoresis. D-Mannose was identified in acid hydrolysates of the reduced polymers by preparation of a characteristic phenylhydrazone derivative (mp 198–200°) and by a specific reaction with hexokinase and adenosine triphosphate (ATP). L-Gulose was identified by reduction to a product that was a substrate for D-sorbitol dehydrogenase.

The isolation of a "mucus-producing" *Pseudomonas* from the respiratory tract of patients with cystic fibrosis has been reported by Doggett *et al.* (1964, 1965). Blood agar, covered by a dialysis membrane, was used as the growth medium, and the viscous material elaborated by these organisms was isolated by an ethanol–benzene method (DiSant'Agnese *et al.*, 1957). The product was identified as a polysaccharide containing glucose, mannose, galactose, glucosamine, galactosamine, sialic acid, and two unidentified compounds. Since this material contained no naphthoresorcinol-reactive material, these authors concluded that no uronic acids were present.

Linker and Jones (1964) subsequently reported on the isolation of another polysaccharide produced by a *Pseudomonas*, also isolated from the respiratory tract of a patient with cystic fibrosis. This material was purified by extraction with dilute alkali, precipitation with cetylpyridinium chloride, and ethanol fractionation. The product resembled alginic acid and mannuronic and guluronic acids were the principal components.

The "mucoid-type" *Pseudomonas* reported by Doggett *et al.* and by Linker and Jones were found only in the tracheobronchial tree of cystic fibrosis patients. The possible significance of this, together with the reported differences in polysaccharide constituents of the viscous materials isolated from these organisms, prompted a further investigation of related *Pseudomonas* strains.

A number of "mucoid-type" *Pseudomonas* organisms were available to us, and all had been identified as *Pseudomonas aeruginosa*.[1] The polysaccharides produced by 13 "mucoid-type" organisms, both from cystic fibrosis and noncystic fibrosis sources, were compared and all were found to be polyuronic acids. The principal component of each polyuronide prepara-

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[1] A manuscript is in preparation describing these organisms as *P. aeruginosa*. The authors wish to thank Dr. Bernard Boxerbaum, Department of Pediatrics, University Hospitals, Cleveland, for making these data available.

tion was D-mannuronic acid. Variable amounts of L-guluronic acid were also identified. Neither neutral sugars nor hexosamines were detectable.

Experimental Procedure

Materials. All materials used were of commercial origin with the exception of D-gulose (Dr. B. A. Lewis, University of Minnesota) and crystalline mannuronolactone (Dr. G. Ashwell, National Institutes of Health). Alginic acid, prepared in the following manner, also was used in these studies. Alginic acid (Aldrich Chemical Co., Milwaukee) was extracted with 10 volumes (w/v) of a methanol–0.05 N HCl solution (2:1, v/v) for 12 hr at 4°. The insoluble residue was removed by filtration, washed with methanol to remove HCl, and dried *in vacuo* over P_2O_5.

D-Sorbitol dehydrogenase was prepared from rat liver (Blakley, 1951). A crude enzyme preparation, containing a mixture of phosphomannose isomerase and phosphoglucose isomerase, was isolated from rat muscle (Slein, 1955).

Methods. Radioactivity was measured in a Model 4312 Packard Tri-Carb liquid scintillation counter using methods recommended by the manufacturer. A Gilford multiple sample recorder was used to follow reduced pyridine nucleotide formation.

The following chromatographic solvent systems and electrophoresis buffers were used: (A) ethyl acetate–pyridine–acetic acid–water (5:5:1:3); (B) 1% sodium tetraborate containing 0.005 M $CaCl_2$; (C) 1% sodium tetraborate; (D) 1-butanol–acetic acid–water (4:1:5); (E) pyridine–ethyl acetate–water (2:5:7); (F) ethyl acetate–pyridine–water (2:1:2); (G) 1-butanol–pyridine–water (6:1:2); (H) ethyl acetate–pyridine–acetic acid–water (5:5:1:5). Descending chromatography was performed using Whatman No. 1 paper. Paper electrophoresis was conducted with Whatman 3MM paper using B and C as buffers. Electrophoresis for 45 min at 45 v/cm in a Gilson high-voltage electrophorator was sufficient for separation of both hexoses and uronic acids. The first borate system B gave better separation of the uronic acids (Haug and Larsen, 1961); the second system C was used for the separation of hexoses. Sugars were detected on paper by the periodate–benzidine technique (Gordon *et al.*, 1956).

The following substances were determined by the indicated methods: reducing sugars according to Park and Johnson (1949); protein by the procedure of Lowry *et al.* (1951); sialic acid by the resorcinol method (Svennerholm, 1958); hexoses by the anthrone procedure (Seifter *et al.*, 1950); fucose by the cysteine–sulfuric acid method of Dische and Shettles (1948); hexosamine by a modified Elson–Morgan procedure (Boas, 1953); phosphorus by the method of Fiske and Subbarow (1925); keto sugars by the method of Cohen (1953); uronic acids by the carbazole procedure of Dische (1947), the orcinol–$FeCl_3$ method of Brown (1946), and the decarboxylation method outlined by Tracey (1948).

Polysaccharide Preparation. *P. aeruginosa* was grown in trypticase soy broth for 12–20 hr in a New Brunswick Gyrotary shaker at 35° and 100 oscillations/min. The cells were harvested by centrifugation at 37,000*g* for 30 min. The viscous supernatant fluid was removed by decanting. In some instances water was added prior to centrifuging to decrease the viscosity which hindered the sedimentation of the cells. Ethanol (2 volumes of 95%) was added to the supernatant fluid and the heavy clot which formed was removed by wrapping it around a stirring rod. The yield (dry weight) of crude polysaccharide varied from 1 to 2 g/l. of culture fluid. No additional clotting was observed after either increasing the ethanol concentration or adding a mixture of ethanol–benzene (DiSant'-Agnese *et al.*, 1959). The clot was dissolved with water to the original volume and the ethanol precipitation repeated using 2 volumes of ethanol saturated with sodium acetate. The ethanol-insoluble material was dissolved in 1 volume of 0.01 N NaOH, stirred at 25° for 4 hr, and reprecipitated by the addition of 2 volumes of ethanol saturated with sodium acetate. This insoluble material was removed as before, washed once with ethanol, dissolved in water, and purified by cetylpyridinium chloride (CPC)[2] precipitation (Scott, 1955). An aqueous solution of CPC was added to a final concentration of 0.1% and the resulting suspension stirred at 37° for 1 hr. The precipitate, removed by centrifuging at room temperature, was washed twice with water and then stirred 4 hr with ethanol saturated with sodium acetate. The residue was removed by centrifuging and again extracted with the ethanol–sodium acetate solution. The insoluble material remaining after the second extraction was dissolved in water and dialyzed against distilled water at 4°. The nondialyzable material was lyophilized. This purification procedure is similar to that used by Linker and Jones (1964). The resulting polysaccharides were stored in the desiccated state at room temperature.

Polysaccharide Analysis. The viscous materials elaborated by all the organisms studied, regardless of source, are polyuronic acids and contain few, if any, other components (Table I). Protein, hexose, phosphorus, hexosamine, deoxy sugar, and sialic acid were not detected. Since there is a difference in the extinction coefficients of chromogens produced from the different uronic acids with the carbazole method (Hoffman *et al.*, 1956), and since large variations were found for the orcinol–$FeCl_3$ results,[3] the more reliable quantitative values are the CO_2 values obtained on decarboxylation (Tracey, 1948). The very compact stringy nature of the dried polysaccharide preparations was such that hydrolysis using the described conditions (5 hr at 115° in 4 N HCl) did not give complete decarboxylation. It was necessary to dry aliquots of a solution of the poly-

[2] Abbreviations used are CPC, cetylpyridinium chloride; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate; P, phosphate.

[3] Dr. A. Linker (personal communication) has also experienced large variations with the orcinol–$FeCl_3$ determination with these polysaccharides.

TABLE I: Analyses of the Polyuronic Acids.

Source[a]	CO_2[b]	Carbazole[c]	Orcinol–$FeCl_3$[c]	Carbazole/Orcinol
		Pulmonary Tract		
Cystic fibrosis				
1	81.1	23.3	61.2	0.38
33	88.4	19.2	65.2	0.29
34	84.6	19.5	67.1	0.29
35	99.5	19.1	50.4	0.38
39	89.7	24.5	81.9	0.30
40	98.4	18.3	71.0	0.26
114[d]	91.9	18.7	33.0	0.57
115[d]	91.4	20.5	75.8	0.27
Noncystic fibrosis				
60	107.6	26.4	75.5	0.35
		Urinary Tract		
Noncystic fibrosis				
52	103.6	14.3	39.0	0.37
57	96.5	20.5	70.9	0.28
58	...	13.5	54.0	0.25
59	80.8	24.6	76.0	0.32

[a] Bacterial cultures obtained from different patients were arbitrarily given numbers. [b] CO_2 is calculated as per cent of theoretical CO_2 content of the sodium salt of anhydrouronic acid (22.4%). [c] Carbazole and orcinol–$FeCl_3$ values are calculated as per cent of dry weight based on a glucuronic acid standard. Mannuronic acid gives 17% the color of glucuronic acid in the carbazole reaction (Hoffman *et al.*, 1956). [d] Cultures received from Mr. R. G. Doggett.

saccharides in tared hydrolysis vessels prior to hydrolysis; two or three portions of the viscous solutions were dried to obtain the necessary weight of polyuronide for hydrolysis. This procedure did undoubtedly introduce additional error into the determinations and may have been the reason for some of the variability found among the samples. All decarboxylations were carried out in duplicate and the values agreed within 5%.

The identification of the constituent uronic acids required hydrolysis of the polyuronides. However, difficulties in hydrolyzing uronic acid containing polymers have been experienced by several workers (Schoeffel and Link, 1932; Danishefsky *et al.*, 1962; Spoehr, 1947) and recoveries were low, presumably because of decarboxylation. Direct attempts to hydrolyze alginic acid and the polyuronides isolated from *Pseudomonas* resulted in a rapid decrease in carbazole-reacting material. While it was possible to isolate sufficient mannuronic acid for identification, following hydrolysis in 88% formic acid (Spoehr, 1947), only small amounts of guluronic acid were found. In some preparations guluronic acid was not detected. The results of electrophoretic and chromatographic analysis of hydrolyzed samples of the polyuronic acids and alginic acid, using the conditions outlined by Spoehr (1947), are given in Table II. While only four polysaccharides are listed, similar results were obtained with the other polysaccharide preparations.

TABLE II: Chromatographic and Electrophoretic Characterization of Hydrolysis Products.

Sample	Solvent System A $R_{\text{mannuronic acid}}$	B $R_{\text{mannuronic acid}}$	C[a] R_{mannose}
Standards			
Mannuronic acid	1.00	1.00	...
Mannurono-lactone	0.54	...	...
Mannose	1.21	...	1.00
Glucose	1.11	...	1.59
Galactose	0.97	...	...
Gulose	...	...	1.34
Polyuronides			
Alginic acid	0.54, 0.99	1.02, 1.24	1.03, 1.34
52	0.54, 0.99	1.00, ...	1.03, ...
60	0.54, 1.00	1.01, 1.27	1.03, 1.37
115	0.54, 1.01	1.05, 1.25	1.00, 1.33

[a] Migrations of sugars obtained from hydrolysis of the reduced polyuronides.

Since acid hydrolysis of the polyuronic acids produced by *Pseudomonas* could possibly result in the selective destruction of guluronic acid, the carboxyl groups of these compounds were reduced to yield the corresponding hexosans. For this procedure, alginic acid and purified preparations of polyuronides 52, 60, and 115 (Table I) were extracted twice with acetone by homogenizing at low speed in a Servall omnimixer, then centrifuged. The residue was dried *in vacuo* at 80° overnight. The methyl esters then were prepared, essentially by the method reported by Jansen and Jang (1946). Each polyuronide was suspended in 100 ml of 0.05 N anhydrous methanolic HCl in 250-ml Corex centrifuge bottles (Ivan Sorvall, Inc., Norwalk, Conn.) and mixed by shaking at 35° for 48 hr. The insoluble material was then harvested by centrifugation, the supernatant fluid was discarded, and the methanolic HCl treatment repeated twice more for a total of 144 hr. The resulting white flocculent material was washed free of HCl by extracting it four times with a total volume of 200 ml of methanol. The weights of the methyl esters are given in Table III. Reduction of the methyl esters was carried out essentially as described for the methyl ester of heparin (Danishefsky *et al.*, 1962). The esters of alginic acid and polyuronides 52, 60, and 115 were dissolved in 10 ml of water, 1.0 ml

TABLE III: Analysis of the Reduced Polyuronides.

	Source of Polysaccharide			
	Alginic Acid	*Pseudomonas*		
		52	60	115
Polyuronide (mg)	290	165	156	192
Methyl ester (mg)	263	132	142	149
Reduced polysaccharide (mg)	253	92	125	112
Anhydrohexose (mg)[a]	100	54	100	85
Uronic acid reduced (%)[b]	40	59	81	77
Reduced polysaccharide hydrolyzed (mg)	173	49	76	63
Recovery as reducing sugar (%)[c]	22	40	47	41
Mannose phenylhydrazone (mp, °C)	199–200	198–202	199–201	199–200
Ratio of mannose:gulose	18:1	...	17:2	18:1

[a] Based on anthrone values using mannose as standard. [b] Weight of anhydrohexose/weight of reduced polysaccharide × 100 = % reduction. [c] Following deionizing of the hydrolyzed samples reducing sugar was measured by the method of Park and Johnson (1949) and calculated as anhydrohexose.

of a 10% solution of sodium borohydride was added, and the resulting mixture was stirred at 4° for 12 hr. The solutions were adjusted to pH 4 with acetic acid and each preparation was dialyzed against 400 ml of distilled water at 4°. The dialysis water, as determined by the carbazole and anthrone methods, contained neither uronic acids nor hexoses. Dialysis was continued against two 400-ml changes of 0.1 M acetic acid and finally against distilled water. The contents of the dialysis bags were removed and the pH adjusted to 7.0 with dilute KOH. A small amount of insoluble material was removed by centrifugation. The clear supernatant fluids were assayed for hexose by the anthrone procedure (Table III).

The indicated amounts (Table III) of each of the reduced polysaccharides in 12.5 ml of 2 N H_2SO_4 were hydrolyzed for 6 hr at 100°. The pH of the hydrolysate was adjusted with saturated barium hydroxide to between 5 and 5.5, the barium sulfate was removed by centrifuging, and the supernatant fluid was deionized by the addition of mixed-bed ion-exchange resin, Bio-Rad AG 501-x8. The yield of reducing sugar was 40–50% for the three *Pseudomonas* polysaccharides and 22% for alginic acid (Table III). Chromatography and electrophoresis (Table II) showed that only mannose was present in the preparation derived from the urinary polyuronide, 52, whereas both mannose and gulose were obtained from polyuronides 60, 115, and alginic acid. A differentiation of gulose and mannose was achieved only by electrophoresis in borate buffer. The sugars obtained from the hydrolysis of the reduced polyuronides migrated with mannose and gulose in solvents D–H. Therefore, while all the viscous materials are polyuronic acids, the nature of the constituent uronic acids seems to vary.

Preparative paper electrophoresis was used to isolate sufficient quantities of the individual hexoses for analysis and identification. Each of the reduced, hydrolyzed polysaccharides was subjected to electrophoresis in 1% sodium tetraborate and the areas corresponding to the migration of mannose and gulose were eluted with water. The solutions were passed through 1 × 5 cm columns of Dowex 50-x-8 H^+, 200–400 mesh, and the eluates evaporated to dryness. Methyl borate was removed by repeated evaporation with methanol.

Mannose was identified by (1) preparing its phenylhydrazone derivative, (2) reducing it to mannitol, and (3) its reaction with hexokinase and ATP. The phenylhydrazone of mannose was prepared as described by Isbell and Frush (1962). The melting points of these derivatives (Table III) are similar to reported values (199–200°) and to values obtained for mannose phenylhydrazone prepared in our laboratory (198–200°). In addition to the chromatographic and electrophoretic characterization, gulose was identified by reduction to D-sorbitol and by the reaction of the reduced product with D-sorbitol dehydrogenase.

Reduction of the sugars, isolated after paper electrophoresis, with sodium borohydride resulted in mannitol and sorbitol as demonstrated by chromatography and electrophoresis. The configurations of the isolated mannose and gulose were shown to correspond to D-mannose and L-gulose by the following criteria. (a) The mannose was active as a substrate for yeast hexokinase, coupled with phosphomannose isomerase, phosphoglucose isomerase, and glucose 6-P dehydrogenase. The details on the assay procedure and the results are given in Figure 1. Neither L-mannose nor the isolated gulose was active with the assay system. (b) The product resulting from the reduction of gulose was active as a substrate for liver D-sorbitol dehydrogenase (Figure 2); L-sorbitol, prepared by reducing D-gulose, was inactive. As the reduction of either L-gulose or D-glucose results in the formation of D-sorbitol, and as both reduced products were active as the substrate for D-sorbitol dehydrogenase, the

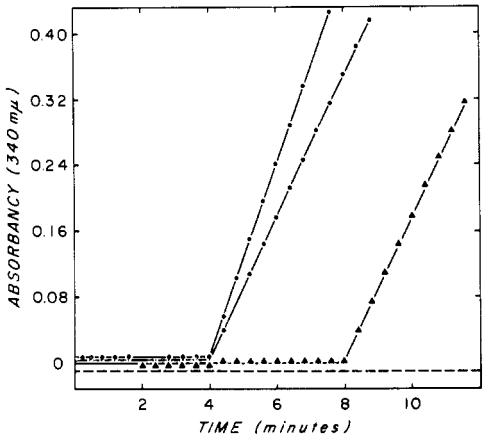

FIGURE 1: Assay method for D-mannose. Incubation mixtures contained the following components (micromoles) in a final volume of 1.0 ml: Tris–hydrochloride buffer, pH 8.0, 40.0; TPN, 0.2; magnesium chloride, 8.0; and glucose 6-P dehydrogenase, 2.5 units. Cuvets contained the following sugars (micromoles): – – – –, control; ▲—▲, L-mannose, 0.25; ●—●, D-mannose, 0.25; O—O, mannose from reduced polyuronide 60, 0.40. The following additions were made to all cuvets at the indicated times: 0.025 ml of hexokinase–ATP mixture (250 units of yeast hexokinase and 100 μmoles of ATP/ml of 0.1 M glycine buffer, pH 8.0), 2 min; 0.05 ml of phosphomannose isomerase (200 units)–phosphoglucose isomerase mixture (see Materials), 4 min. After incubating for 8 min, 0.25 μmole of D-mannose was added to the cuvet containing L-mannose.

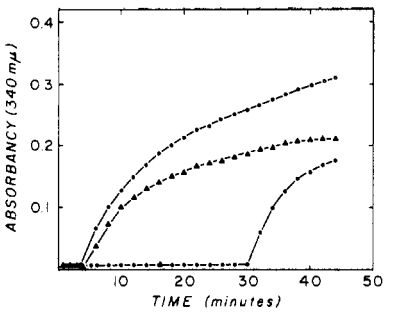

FIGURE 2: Assay procedure for D-sorbitol. Incubation mixtures contained the following components (micromoles) in a final volume of 1.0 ml: Tris–hydrochloride buffer, pH 8.0, 40.0; DPN, 1.0; 200 units of D-sorbitol dehydrogenase; and the indicated amounts (micromoles) of the reduced substrates (see text). ●—●, mannose isolated from reduced polyuronide of *Pseudomonas* 115, 0.1; ▲—▲, gulose isolated from reduced alginic acid, 0.1; and O—O, gulose isolated from reduced polyuronide from *Pseudomonas* 115, 0.13. After incubating for 30 min, 0.1 μmole of D-sorbitol was added to the cuvet containing the mannose.

gulose obtained from the reduced polyuronide was assumed to be in the L configuration. Fructose, the product of the D-sorbitol dehydrogenase reaction, was recovered in stoichiometric amounts as determined by the keto sugar assay of Cohen (1953). These data suggest that the uronic acids in the polyuronides are D-mannuronic acid and L-guluronic acid.

In view of the difference found in the guluronic acid content of the four polyuronides described, it was necessary to study the remaining polysaccharide preparations. The polyuronides listed in Table I, including preparations of *Pseudomonas* 52, 60, and 115 different from those used previously, were reduced and the resulting polysaccharides hydrolyzed and deionized as described above. Electrophoresis in borate buffer indicated that the principal sugar in every preparation was mannose, but that gulose was detectable in all samples, including the polyuronide from *Pseudomonas* 52. While the mannose-to-gulose ratio was not quantitatively determined for each preparation, it was evident from the relative intensities of spots on electrophoretograms that the proportions of the two sugars differed in polyuronides isolated from the various sources. The discrepancy with the previous experiment, where gulose was not detected in a polyuronide from *Pseudomonas* 52, is not as yet understood, but the ability of these organisms to synthesize guluronic acid, or its polymer, may change after repeated transfer of the cultures. For example, the ability of *Pseudomonas* 60 to produce mucoid colonies was lost entirely after several transfers. In addition to mannose and gulose, the reduced, hydrolyzed polyuronides also contained a trace compound which migrated slightly slower than mannose in borate electrophoresis (R_{mannose} 0.92). While this material was not identified, sodium borohydride reduction yielded a compound which migrated with sorbitol (R_{mannose} 1.38).

[^{14}C]*Glucose Incorporation. Pseudomonas* organisms are capable of oxidizing D-glucose to D-gluconic acid (Stokes and Campbell, 1951). The oxidation of C-6 of D-gluconic acid to an aldehyde would result in the formation of L-guluronic acid. Reactions involving an oxidation of this type (Bernhauer and Irrgang, 1935) and the phosphorylation of a uronic acid (Neufeld *et al.*, 1959) have been described. It was of interest, therefore, to incorporate specifically labeled glucose into a polyuronide, in an attempt to demonstrate whether inversion of the carbon chain occurred.

[^{14}C]Glucose was added to growing cultures of *Pseudomonas* 1 (Table I). [1-^{14}C]Glucose and [6-^{14}C]-glucose (1 μc each) were added to separate 20-ml aliquots of a 10-hr culture and incubation was continued for 6 hr. The cells were removed by centrifuging and the polysaccharides were clotted by the addition of 2 volumes of 95% ethanol. The resulting clots were repeatedly dissolved in water and reprecipitated as before, until the supernatant fluids contained no radioactivity. The alcohol-insoluble materials, which contained about 200,000 cpm from each radioactive source, were dried and decarboxylated (Tracey, 1948).

The CO_2 liberated was collected in 2 ml of a 1:1 mixture of ethanolamine–Methyl Cellosolve and counted: 160,000 cpm from [6-^{14}C]glucose and 1000 cpm from [1-^{14}C]glucose. While not conclusive, these data suggest that glucose is incorporated into the polyuronic acid without randomization or inversion of the carbon chain.

Discussion

The results reported in this study confirm and extend the findings of Linker and Jones (1964) in that uronic acids were the only detectable components of polysaccharides elaborated by the "mucoid-type" *Pseudomonas* isolated from cystic fibrosis patients. These organisms, however, are found not only in the pulmonary tract of cystic fibrosis patients but also in the pulmonary and urinary tracts of noncystic fibrosis patients.[1]

Polysaccharides isolated from the "mucoid-type" *Pseudomonas*, regardless of source, have been shown to be polyuronic acids.[4] Variable amounts of guluronic acid were found in different polyuronide preparations. With the evidence available, it is not possible to determine whether both uronic acids are present in the same molecule, a heteropolymer, or if two separate polyuronic acids exist, one of mannuronic acid and the other guluronic acid. The isolation of a polysaccharide containing only mannuronic acid suggests the presence of two homopolymers.

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[4] During the preparation of this manuscript, the presence of *O*-acetyl groups in polyuronic acids isolated from *P. aeruginosa* was reported (Linker and Jones, 1966).

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