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SEPARATION OF SIALYL-OLIGOSACCHARIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO ANALYSIS OF CARBOHYDRATE UNITS OF ACIDIC OLIGOSACCHARIDES OBTAINED BY HYDRAZINOLYSIS OF HEN OVOMUCOID

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SUMMARY

Sialyl-oligossacharides derived from hen ovomucoid by hydrazinolysis have been separated by liquid chromatography on quaternary amine bonded silica and alkylamine modified silicas. By using a mobile phase consisting of a mixture of acetonitrile and potassium dihydrogen phosphate with 0.01% of 1,4-diaminobutane, effective resolution of high-molecular-weight monosialylated oligossacharides was achieved in less than 2 h.

INTRODUCTION

Hen ovomucoid is a glycoprotein with a molecular weight of 28,000 and carbohydrate content of 25% which plays a role in the inhibitory activity proteolytic enzymes[1]. The glycoprotein is characterized by a high degree of microheterogeneity of its carbohydrate moieties[2,3]. Because of the difficulty of obtaining homogeneous glycopeptides, few investigations have been made of the structures of the carbohydrate fractions of ovomucoid. Montreuil and co-workers studied extensively the structure of asialo-ovomucoid by partial hydrolysis[4], acetolysis[5] and methylation analysis[6] and presented a complete structure[7]. Yamashita *et al.*[8] isolated, by hydrazinolysis and chromatography on Bio-Gel P_4, eight fractions and showed that penta-antennary complex type asparagine-linked sugar chains occur in a pure glycoprotein. In a later paper[9] they reported the results of structural studies of the remaining smaller oligosaccharides and indicated that the sugar chains of hen ovomucoid may be synthesized by a pathway different from the general processing pathway. Conchie and co-workers[10–12] established on the asialo-ovomucoid glycopeptides the existence of the unusual feature of two different, triply-substituted mannosyl residues occurring in a single glycopeptide.

We have described[13] the preparation by high-performance liquid chromato-

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graphy (HPLC) on a bonded-phase amine column of 17 fractions of oligosaccharide alditols obtained by hydrazinolysis, N-reacetylation and reduction of asialo-ovomucoid. The primary structural analyses of the major fractions were conducted by applying 500 MHz ^{1}H NMR spectroscopy in combination with methylation analysis[14,15] and mass spectrometric analysis[16]. Novel types of asparagine-bonded carbohydrate chain were determined and appeared to consist of an intersected penta-antennary structure with zero, one and two galactose residues.

We describe here an HPLC method for the separation of acidic oligosaccharides obtained by hydrazinolysis of hen ovomucoid on bonded primary amine packings with solvents containing 1,4-diaminobutane.

EXPERIMENTAL

Glycoprotein and oligosaccharides

Ovomucoid was prepared according to Fredericq and Deutsch[17]. Oligosaccharides were released from 900 mg of ovomucoid by hydrazinolysis as previously described[18]. The resulting oligosaccharides were N-reacetylated according to Reading *et al.*[19] and reduced with potassium borohydride.

Purification of oligosaccharides by gel filtration

A 1-mg amount of oligosaccharides was N-reacetylated with [^{14}C]acetic anhydride and reduced with potassium borohydride. Oligosaccharides were purified by gel filtration on Bio-Gel P-2 (200–400 mesh) (Bio-Rad Labs.) columns (92 × 2 cm I.D.), eluting with 0.5 *M* acetic acid at a flow-rate of 20 ml/h. The volume of each fraction was 4 ml. The total oligosaccharides liberated from ovomucoid by hydrazinolysis were subjected to gel filtration chromatography under similar conditions.

Separation of oligosaccharides from Bio-Gel P_2 fractions of hydrazinolysate of hen ovomucoid by HPLC on quaternary amine bonded silica[20]

HPLC was performed on a 10-μm Micro-PAK AX-10 column (50 × 0.8 cm I.D.; Varian) with a Spectra-Physics Model 700 liquid chromatograph equipped with Model 8400 variable-wavelength detector connected to a Model 4100 computing integrator.

For preparative chromatography, 20 mg of Bio-Gel P2 fraction (26–33) dissolved in 60 μl of distilled water were subjected to HPLC using a gradient of 500 m*M* potassium dihydrogen phosphate (KH_2PO_4) (adjusted to pH 4.0 with phosphoric acid) as follows: elution with distilled water for 15 min, linear gradient to 2.5% KH_2PO_4 (500 m*M*) for 10 min, isocratic elution for 20 min, linear gradient to 5% KH_2PO_4 (500 m*M*) for 10 min, isocratic elution for 20 min and linear gradient to 40% KH_2PO_4 (500 m*M*) for 40 min. The flow-rate was 2 ml/min. The oligosaccharides were detected at 200 nm with a detector sensitivity 0.32 and integrator attenuation 16. The chart speed of the integrator was 0.5 cm/min.

For semi-preparative chromatography, 1 mg of Bio-Gel P_2 fraction (34–44) dissolved in 20 μl of distilled water was subjected to HPLC using a gradient of 500 m*M* potassium dihydrogen phosphate (adjusted to pH 4.0 with phosphoric acid) as follows: elution with distilled water for 25 min, linear gradient to 2.5% KH_2PO_4 (500 m*M*) for 10 min, isocratic elution for 10 min, linear gradient to 5% KH_2PO_4 (500

m*M*) for 10 min, isocratic elution for 10 min, linear gradient to 40% KH_2PO_4 (500 m*M*) for 40 min. The oligosaccharides were detected at 200 nm with a detector sensitivity of 0.16 and integrator attenuation 16. The chart speed of the integrator was 0.5 cm/min.

Each fraction, except neutral oligosaccharides F-I and F-A eluted with water, was purified by gel filtration on Bio-Gel P-2 as previously described. The fractions were revealed with orcinol–sulphuric acid reagent[21] on silica gel plates (pre-coated silica gel 60; Merck). The phosphate salts eluted from the Bio-Gel column were identified by precipitation with silver nitrate.

Separation of monosialyl-oligosaccharides (F-II) by liquid chromatography on primary amine bonded silica

HPLC was performed on three columns in series: two columns of 5-μm Amino AS 5A (25 × 0.4 and 15 × 0.4 cm I.D., Chromatem 33; Touzard et Matignon) and one column of 3-μm amino column (7.5 × 0.4 cm I.D., Chromatem 33; Touzard et Matignon). For semi-preparative chromatography 2 mg of oligosaccharides dissolved in 20 μl of 50 m*M* potassium dihydrogen phosphate containing 0.01% of 1,4-diaminobutane (DAB) were injected into the column. The column was equilibrated with the initial solvent (solvent A), consisting of a mixture of acetonitrile (70%) and a 50 m*M* potassium dihydrogen phosphate containing 0.01% of 1,4-diaminobutane (30%). After the injection, isocratic conditions were applied for 30 min with solvent A, followed by a linear gradient to solvent A–water (85:15) for 60 min and then isocratic conditions for 60 min. The flow-rate was 1 ml/min. The oligosaccharides were detected at 200 nm with a detector sensitivity of 0.16 and integrator attenuation 16. The chart speed of the integrator was 0.5 cm/min.

Each collected fraction containing oligosaccharides, potassium dihydrogen phosphate and 1,4-diaminobutane was purified by rapid gel filtration on Bio-Gel P-6 DG (Bio-Rad Labs.; 90–180 μm; 25 × 1.6 cm I.D. column) and a Spectra-Physics Model 8770 liquid chromatograph with a 500-μl sample loop. Oligosaccharides were detected with a UV detector (LKB 2138 Uvicord S) at 206 nm. The flow-rate of distilled water was 0.4 ml/min.

Molar composition of oligosaccharides

The molar composition of oligosaccharides was determined by gas–liquid chromatography (GLC) of trifluoroacetylated methylglycosides according to Zanetta *et al.*[22].

RESULTS AND DISCUSSION*

The separation of a mixture of reduced N-[^{14}C]acetylated oligosaccharides obtained by hydrazinolysis from hen ovomucoid on a Bio-Gel P-2 column is shown in Fig. 1. The purification of the hydrazinolysate of 900 mg of hen ovomucoid gives two fractions: 210 mg of F(26–33), and 5 mg of F(33–44). The yield of oligosaccharides was 95.5%. The carbohydrate composition of these fractions was determined

* Abbreviations: Gal = galactose; Man = mannose; GlcNAc = N-acetylglucosamine; GlcNAc-ol = N-acetylglucosaminitol; NeuAc = N-acetylneuraminic acid.

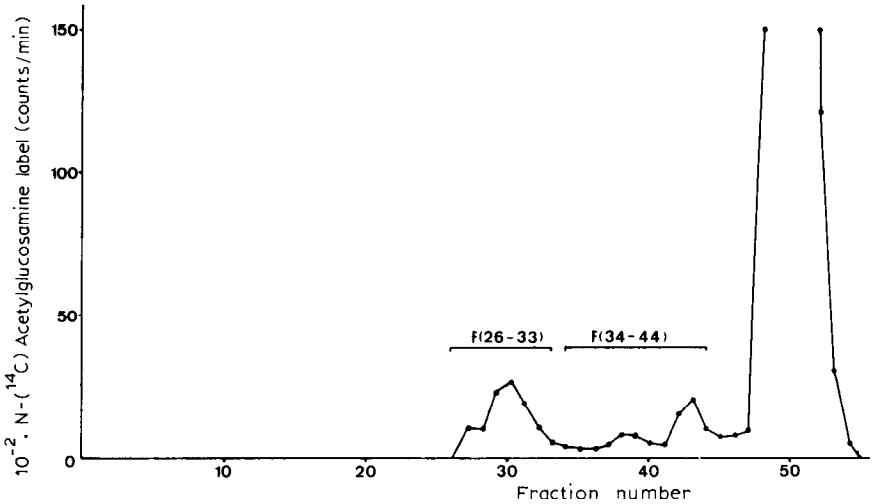

Fig. 1. Gel filtration of oligosaccharides liberated from hen ovomucoid by hydrazinolysis on Bio-Gel P-2.

by GLC (Table I). The major fraction (F26–33) is characterized by a high GlcNAc/Man ratio (2.01) already found in total ovomucoid by Conchie and Hay[11] and by our group in oligosaccharides from the hydrazinolysis of hen ovomucoid neutral glycopeptides[13]. The minor fraction (F33–44) is characterized by a high content of sialic acid with a low GlcNAc/Man ratio (1.46).

HPLC on quaternary amine packings of the major fraction F(26–33) obtained by Bio-Gel P-2 chromatography of the hydrazinolysate of hen ovomucoid gives excellent separations of oligosaccharides containing zero, one, two, three and four sialic acid residues (Fig. 2). Eight fractions were obtained within 120 min. The results of the preparative chromatography of 210 mg of ovomucoid-derived oligosaccharides F(26–33) and the carbohydrate compositions of the fractions are given in Table II. The use of a 50 × 0.8 cm I.D. column filled with silica modified by an organic quaternary amine provides a quantitative recovery (98.64%) of the oligosaccharides. The major fraction is consisted of neutral oligosaccharides (F-I, 82.3%), and for the sialylated part of ovomucoid (17.65%) the monosialylated fraction (F-II) represented

TABLE I

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY BIO-GEL P-2 CHROMATOGRAPHY OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS OF HEN OVOMUCOID

Fractions	*Molar ratio**				*GlcNAc / Man*	*Weight (mg)*
	Gal	*Man*	*GlcNAc*	*NeuAc*		
Ovomucoid	0.67	3	6	0.07	2	900
F(26–33)	0.67	3	6.04	0.07	2.01	210
F(34–44)	0.45	3	3.95	0.43	1.31	5

* The molar ratio of mannose (Man) was taken as 3.

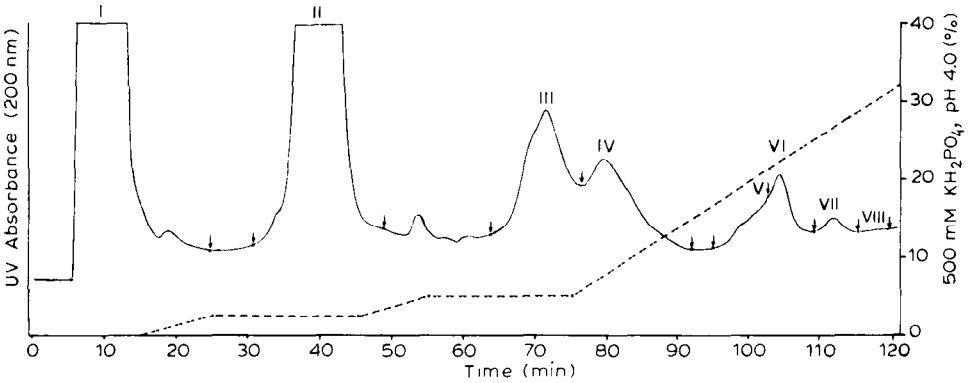

Fig. 2. Analysis of oligosaccharides from the hydrazinolysis of hen ovomucoid F(26–33) on 10-μm AX-10.

TABLE II

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY PREPARATIVE CHROMATOGRAPHY OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS OF HEN OVOMUCOID F(26–33)

Fractions	*Molar ratio**					*GlcNAc + GlcNAc-ol / Man*	*Weight (mg)*
	Gal	*Man*	*GlcNAc*	*GlcNAc-ol*	*NeuAc*		
Total oligosaccharides, F(26–33)	0.67	3	6.04		0.07	2.01	210
Neutral oligosaccharides, F-I	0.49	3	5.68	0.27	0	1.98	170.59
Monosialyl-oligosaccharides, F-II	1.10	3	4.62	0.40	1	1.67	27.17
Disialyl-oligosaccharides, F-III	2.15	3	4.88	0.27	2	1.71	5.79
Disialyl-oligosaccharides, F-IV	1.96	3	5.86	0.46	2	2.10	1.30
Trisialyl-oligosaccharides, F-V	2.85	3	4.72	0.50	3	1.74	0.46
Trisialyl-oligosaccharides, F-VI	3.1	3	5.16	0.37	3	1.84	1.36
Tetrasialyl-oligosaccharides, F-VII	3.9	3	5.55	0.52	4	2.02	0.37
Tetrasialyl-oligosaccharides, F-VIII	3.95	3	4.53	0.71	4	1.74	0.11
Recovered							207.15

* The molar ratio of mannose (Man) was taken as 3.

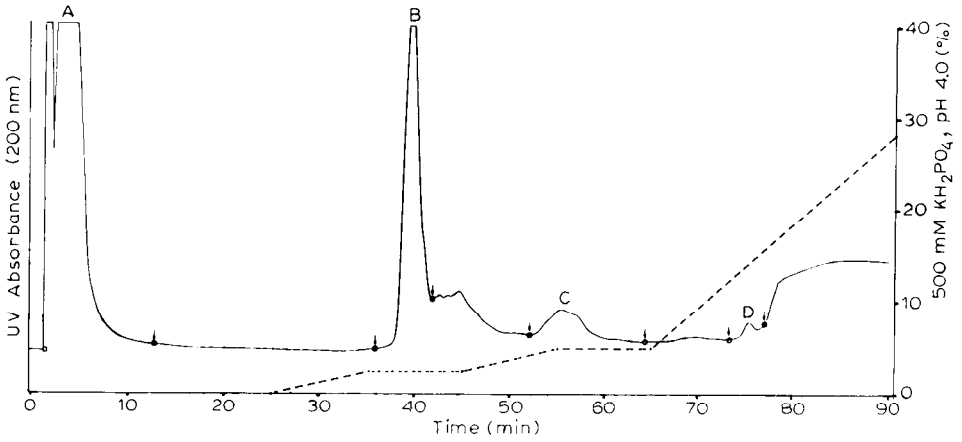

Fig. 3. Analysis of oligosaccharides from the hydrazinolysis of hen ovomucoid F(34 44) on 10-μm AX-10.

13.1% of total ovomucoid. These results are in agreement with values found by Yamashita *et al.*[8] by paper electrophoresis of oligosaccharides released by hydrazinolysis from hen ovomucoid (85% for the neutral component). Except for the monosialylated fraction, each acidic fraction gives two classes of compounds separated according to the number of branches linked to the trimannosido core, which is related to the GlcNAc + GlcNAc-ol/Man ratio. The monosialylated fraction (F-II) was

TABLE III

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY PREPARATIVE CHROMATOGRAPHY OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS OF HEN OVOMUCOID F(34 44)

Fractions	*Molar ratio**					*GlcNAc + GlcNAc-ol / Man*	*Weight (μg)*
	Gal	*Man*	*GlcNAc*	*GlcNAc-ol*	*NeuAc*		
Total oligosaccharides, F(34 44)	0.45	3	3.95		0.43	1.31	5000
Neutral oligosaccharides, F-A	0	3	3.4		0	1.13	1840
Monosialyl-oligosaccharides, F-B	1	3	2.60		1	0.86	886
Disialyl-oligosacharides, F-C	1.95	3	2.80	0.33	2	1.04	972
Oligosaccharides, F-D	6.09	3	7.5		5.83	2.50	50
Recovery							3748

* The molar ratio of mannose (Man) was taken as 3.

further submitted to HPLC on an alkylamine column with acetonitrile–potassium dihydrogen phosphate–diaminobutane as the eluent.

HPLC of oligosaccharides F(34–44) was performed on a Micropak AX-10 column (Fig. 3). Four fractions were obtained and the carbohydrate composition and weight of each fraction were determined by GLC (Table III). Table III shows that the neutral oligosaccharides fraction (F-A), the monosialylated oligosaccharides fraction (F-B) and the disialylated oligosaccharides fraction (F-C) contain few oligosaccharides with a low GlcNAc + GlcNAc-ol/Man ratio. In contrast, oligosaccharides fraction F-D shows a surprising carbohydrate composition: Gal 6.09, Man 3, GlcNAc 7.5 and NeuAc 5.83 with a high GlcNAc/Man ratio of 2.50, similar to that described for sialylated penta-antennary oligosaccharides by François-Gérard *et al.*[23] in turtledove ovomucoid.

The monosialylated fraction (F-II) from the AX-10 chromatography of oligosaccharides F(26–33) was subjected to HPLC using acetonitrile–potassium dihydrogen phosphate–diaminobutane and alkylamine-modified silicas. The effective separation of seventeen fractions was obtained in 90 min (Fig. 4). The carbohydrate composition and weight of each fraction are given in Table IV. Four major fractions were obtained: oligosaccharides F II-11, F II-12, F II-16 and F II-17. The oligosaccharide F II-11 possesses the same carbohydrate composition as neutral oligosaccharide 6 obtained by HPLC on an alkylamine column of neutral oligosaccharides obtained by hydrazinolysis of hen ovomucoid[13] with additional galactose and sialic acid residues. The oligosaccharide F II-12 is an extension of hen ovomucoid neutral oligosaccharide 7[13,15] and N-5b[9] or chicken ovotransferrin glycopeptide[24] by one residue of galactose and N-acetylneuraminic acid. The oligosaccharide F II-16, with seven GlcNAc residues corresponds to a penta-antennary oligosaccharide like neutral oligosaccharide 14[13,15] and N-2[8] with an additional sialic acid residue. The oligosaccharide F II-17 is a sialylated isomer of N-3a oligosaccharide described by Yamashita *et al.*[9] in neutral oligosaccharides obtained by hydrazinolysis of hen ovomucoid.

Until now, the separation of sialyloligosaccharides was performed on alkylamine bonded columns: oligosaccharides and glycopeptides extracted from the urine of patients with lysosomial diseases with acetonitrile–sodium acetate buffer (pH 5.8)

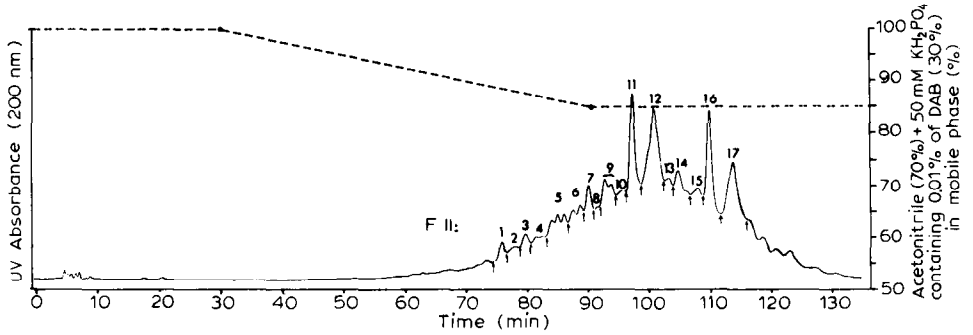

Fig. 4. Analysis of monosialyl oligosaccharides from the hydrazinolysis of hen ovomucoid F-II in Fig. 2 by liquid chromatography on primary amine bonded silica with acetonitrile–potassium dihydrogen phosphate–diaminobutane as eluent.

TABLE IV

CARBOHYDRATE COMPOSITIONS AND WEIGHTS OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF MONOSIALYL-OLIGOSACCHARIDES (F-II) LIBERATED BY HYDRAZINOLYSIS OF HEN OVOMUCOID

Fractions	*Molar ratio**					*GlcNAc + GlcNAc-ol / Man*	*Weight (μg)*
	Gal	*Man*	*GlcNac*	*GlcNAc-ol*	*NeuAc*		
Total monosialyl-oligosaccharides, F-II	1.10	3	5		1	1.66	18,000.0
F-II-1	0.90	3	3.99	0.20	1	1.39	258.76
F-II-2	0.99	3	3.97	0.20	1	1.39	226.29
F-II-3	0.90	3	3.46	0.037	1	1.16	286.95
F-II-4	0.95	3	3.90	0.25	1	1.38	623.02
F-II-5	0.95	3	3.47	0.024	1	1.16	1281.41
F-II-6	0.95	3	4.50	0.36	1	1.62	395.40
F-II-7	1.08	3	5.57	0.09	1	1.88	912.38
F-II-8	1.05	3	6.54	0.512	1	2.35	285.50
F-II-9	0.92	3	3.54		1	1.18	2186.65
F-II-10	1.10	3	5.13	0.20	1	1.77	564.28
F-II-11	1.00	3	4.25	0.85	1	1.70	1726.00
F-II-12	1.09	3	5.11	0.818	1	1.97	2422.00
F-II-13	1.20	3	4.85	0.16	1	1.67	529.28
F-II-14	1.25	3	5.91	0.12	1	2.01	1562.84
F-II-15	1.20	3	4.72	0.15	1	1.62	663.88
F-II-16	1.07	3	6.96		1	2.32	966.36
F-II-17	1.12	3	5.72	0.84	1	2.18	1070.00
Recovery							15,961.05

* The molar ratio of mannose (Man) was taken as 3.

(11:9, v/v)[25], mucine-derived saccharides with linear-gradient elution acetonitrile–phosphate buffer (pH 5.2) (4:1–2:3, v/v)[26,27], alkali-labile sialylated oligosaccharides from Cad glycophorin A[28] with the chromatographic conditions described by Bergh *et al.*[26], cervical mucus oligosaccharides with acetonitrile–water containing 25 m*M* ammonium hydrogen carbonate[29]. Using an anion-exchange resin with a concave gradient of aqueous sodium chloride, Tsuji *et al.*[30] separated sugar chains of porcine and bovine submaxillary mucins, human glycophorin A and porcine thyroglobulin. The procedure described herein allows the fractionation of sialo-oligosaccharides of higher molecular weight. This study has shown that when the mobile phase contains only potassium dihydrogen phosphate in acetonitrile, the separation of high-molecular-weight oligosaccharides containing neuraminic acid is not effective. The addition of 0.01% of 1,4-diaminobutane to the potassium dihydrogen phosphate solution increases the separation of these oligosaccharides on primary amino bonded silica. Under the same conditions, neutral oligosaccharides derived from dolichol-linked oligosaccharide intermediates have been separated by Turco[31] on silica with 0.05% 1,4-diaminobutane in the mobile phase.

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