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COMPARISON OF GEL PERMEATION AND ION-EXCHANGE CHROMATOGRAPHIC PROCEDURES FOR THE SEPARATION OF HYALURONATE OLIGOSACCHARIDES

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SUMMARY

For the separation of hyaluronate oligosaccharides, gel permeation chromatography on Sephadex G-25 and ion-exchange chromatography on Dowex 1-X8 (formate form), DEAE Sephacel (chloride, acetate and formate forms) and Trisacryl M (acetate and formate forms) were compared. Best results were obtained from DEAE Sephacel (formate form) and Dowex 1-X8 (formate form). Even- and odd-numbered hyaluronic acid oligosaccharides up to decasaccharide were well separated. Contaminations were detected by high-performance liquid chromatography.

INTRODUCTION

In the course of studying the enzymatic effect of different glycanohydrolases on hyaluronic acid, it became necessary to prepare substantial amounts of even- and odd-numbered hyaluronate oligosaccharides.

The unbranched glycosaminoglycan hyaluronic acid consists of the basic disaccharide unit N-acetylhyalobiuronic acid (β-D-glycopyranuronosyl-1 → 3-O-(2-acetamido-2-desoxy-β-D-glycopyranosyl)-1 → 4) and is initially hydrolysed by endoglycanohydrolases such as bovine testicular hyaluronidase (E.C. 3.2.1.35) and leech hyaluronidase (E.C. 3.2.1.36). Bovine testicular hyaluronidase cleaves the β-N-acetylglucosaminidic bonds in hyaluronic acid to give a homologous series of even-numbered oligosaccharides with glucuronic acid in a terminal non-reducing position[1]. Leech hyaluronidase also produces even-numbered oligosaccharides but, in contrast, it cleaves hyaluronic acid in oligosaccharides to give the reverse terminal sugar composition with β-N-acetylglucosamine in a terminal non-reducing position[2]. Further degradation requires the use of exoglycanohydrolases, such as β-D-glucuronidase (E.C. 3.2.1.31) or β-N-acetyl-D-glucosaminidase (E.C. 3.2.1.30) to give odd-numbered oligosaccharides with terminal N-acetylglucosamine[3] or glucuronic acid[4].

The oligosaccharide mixtures obtained have been fractionated in a number of

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ways. Adsorption chromatography on Darco celite[5], gel permeation chromatography on Biorad P2, P4, P30[4,6,7], Sephadex G-25 and G-50[11], and ion-exchange chromatography on Dowex 1-X2[12], Dowex 1-X8[13], Dowex 1-X10[1], Dowex 21 K[14] and DEAE cellulose (chloride form)[6] with stepwise and gradient elution have all been used.

In spite of the obvious advantages of using ion-exchange chromatography, the method suffers from poor recovery of oligosaccharides. Furthermore, our own experiments on Dowex 1-X8 (formate form) showed incomplete separation of saccharides when we took high-performance liquid chromatography (HPLC) as the purity criterion. The present report is concerned with the application of ion-exchange chromatography and gel permeation chromatography to the optimal separation and recovery of oligosaccharides derived from hyaluronic acid by enzymatic degradation.

EXPERIMENTAL

Materials

Samples of hyaluronic acid from umbilical cords and leech hyaluronidase were prepared as described previously[15,16]. Bovine testicular hyaluronidase was purchased from Serva (Heidelberg, F.R.G.). Sephadex G-25 and DEAE Sephacel were obtained from Pharmacia (Freiburg, F.R.G.), DEAE Trisacryl M from LKB (Bromma, Sweden) and Dowex 1-X8 resin from Serva.

All other chemicals and solvents used were obtained from commercial sources and were of analytical reagent grade.

Abbreviations

All saccharides labeled with an asterisk are saccharides with terminal reducing glucuronic acid obtained from leech hyaluronidase digestion of hyaluronic acid.

Glucuronic acid (GlcUA); N-acetylglucosamine (GlcNAc); hyaluronic acid (HA).

Even-numbered oligosaccharides obtained by digestion with bovine testicular hyaluronidase: disaccharide (βGlcUAl–3GlcNAc), tetrasaccharide (βGlcUAl–3βGlcNAcl–4)$_2$, hexasaccharide (βGlcUAl–3βGlcNAcl–4)$_3$, octasaccharide (βGlcUAl–3βGlcNAcl–4)$_4$.

Even-numbered hyaluronate oligosaccharides obtained by digestion with leech hyaluronidase: tetrasaccharide* (βGlcNAcl–4βGlcUAl–3)$_2$, hexasaccharide* (βGlcNAc1–4βGlcUAl–3)$_3$, octasaccharide* (βGlcNAcl–4βGlcUAl–3)$_4$.

Odd-numbered hyaluronate oligosaccharides obtained by digestion with bovine testicular hyaluronidase and β-D-glucuronidase: trisaccharide (βGlcNAcl–4βGlcUAl–3βGlcNAc), pentasaccharide (βGlcNAcl–4βGlcUAl–3βGlcNAc1–4βGlcUA1-3GlcNAc), heptasaccharide (βGlcNAcl–4βGlcUAl–3βGlcNAcl–4βGLcUA1–3βGlcNAcl–4βGlcUAl–3GlcNAc).

Odd-numbered hyaluronate oligosaccharides obtained by digestion with leech hyaluronidase and β-N-acetyl-D-glucosaminidase: trisaccharide* (βGlcUAl–3βGlcNAcl–4GlcUA), pentasaccharide* (βGlcUAl–3βGlcNAcl–4βGlcUAl–3βGlcNAcl–4GlcUA), heptasaccharide* (βGlcUAl–3βGlcNAcl–4βGlcUAl–3βGlcNAcl–4βGlcUAl–3βGlcNAc1–4GlcUA).

Preparation of oligosaccharides

HA oligosaccharides were produced by digesting HA of umbilical cords with the appropriate amount of hyaluronidase. All incubations were performed at 37°C.

Even-numbered oligosaccharides with terminal non-reducing glucuronic acid were obtained by digestion of HA with bovine testicular hyaluronidase, as reported previously[15]. Digestion of tetra-, hexa- and octasaccharide with β-N-acetyl-D-glucosaminidase free β-D-glucuronidase from *Patella barbara* resulted in tri-, penta- and heptasaccharide with GlcNAc at terminal residues[16]. Even-numbered oligosaccharides with terminal non-reducing GlcNAc were produced by using leech hyaluronidase[16]. Odd-numbered oligosaccharides with terminal glucuronic acid were prepared with β-D-glucuronidase free β-N-acetyl-D-glucosaminidase of bovine spleen[17]. The reaction products were separated by chromatography on Dowex 1-X8 (formate form) and DEAE Sephacel (formate form) with a linear gradient of formic acid[9]. The purity of the oligosaccharides was checked by HPLC.

Sephadex G-25 gel permeation chromatography

Sephadex G-25, pre-swollen in 0.2 *M* acetic acid or 1 *M* sodium chloride, was packed after decantation and degassing into 160 × 1.8 cm I.D. chromatography columns. Equilibration was performed with eluents overnight. Sample of tetrasaccharide (50 mg) and hexasaccharide (50 mg) were applied to the calibrated Sephadex G-25 columns, and 2-ml fractions were collected and tested with the carbazole reaction for GlcUA-containing saccharides. In all the experiments described, oligosaccharide fractions were pooled and lyophilized for purity testing and concentration by HPLC.

Ion-exchange chromatography on DEAE Sephacel (chloride form)

Tetrasaccharide (50 mg) and hexasaccharide (50 mg) were subjected to chromatography on a 20 × 1.4 cm I.D. column of DEAE Sephacel (chloride form). Elution was accomplished with a linear gradient of 500 ml of distilled water and 500 ml of 0.1 *M* sodium chloride, and 5.6-ml fractions were collected and assayed for GlcUA[18].

Ion-exchange chromatography on DEAE Trisacryl M and DEAE Sephacel converted into the formate form

DEAE Trisacryl M and DEAE Sephacel were pretreated in the same manner: 100 ml of material were washed three times with 500 ml of 0.1 *M* hydrochloric acid and allowed to settle in order to decant fine particles. After washing with distilled water to neutral pH, three 500-ml volumes of 0.1 *M* sodium hydroxide were added and the material was washed again with distilled water to neutrality. Conversion into the chloride form was carried out with three 500-ml of 1.0 *M* sodium chloride. After it had been washed free from chloride, the material was converted into the formate form by washing with 1 *M* sodium formate until the washings gave no precipitation with acidified silver nitrate solution. Excess sodium formate was removed by washing with distilled water.

Tetrasaccharide (50 mg) and hexasaccharide (50 mg) were applied to a DEAE Trisacryl M and to a DEAE Sephacel (both formate form) column (20 × 1.4 cm I.D.). Oligosaccharides were desorbed with a linear gradient of formic acid, rising

from 0 *M* to 0.5 *M* formic acid (total volume 1000 ml). Fractions of 5.6 ml were examined by the carbazole method and HPLC.

Ion-exchange chromatography on DEAE Trisacryl M and DEAE Sephacel converted into the acetate form

Resins were pretreated as described above, but instead of 1 *M* sodium formate a 1 *M* solution of sodium acetate was used. The same amounts of oligosaccharides were chromatographed on 20 × 1.4 cm I.D. columns with a linear gradient of 500 ml of distilled water and 500 ml of 6.0 *M* acetic acid in the case of DEAE Trisacryl M, and 3.0 *M* acetic acid in case of DEAE Sephacel material. Fractions of 5.6 ml were assayed as described above.

Ion-exchange chromatography on Dowex 1-X8 (formate form)

The Dowex 1-X8 (chloride form) resin was pretreated and converted into the formate form according to Murphy and Ranjeker[19]. A 20 × 1.4 cm I.D. column filled with Dowex 1-X8 (formate form) resin was prepared, and oligosaccharides were desorbed with a linear formic acid gradient from 0 *M* to 1.0 *M* formic acid (total volume 1000 ml) and assayed as described above.

Analytical methods

GlcUA was determined by the modified carbazole procedure of Bitter and Muir[20] with D-glucuronic acid-δ-lactone and tetrasaccharide as standards. The purity and concentration of oligosaccharides were checked by HPLC[18]. An HPLC system consisting of a 110 A liquid chromatograph pump, an Altex 210 injection valve, a 5-μl loop (Beckman, Berkeley, U.S.A.), an 2138 Uvicord S UV detector at 206 nm (LKB, Bromma, Sweden) and a C-R1A Chromatopac integrator (Shimadzu, Koyoto, Japan) was used. A 40 × 4.6 mm I.D. LiChrosorb NH_2 column (Dr. H. Knauer, Bad Homburg, F.R.G.) and a 250 × 4.6 mm I.D. Ultrasil NH_2 column (Beckman) were employed. The mobile phase was 0.1 *M* KH_2PO_4 (pH 4.75) and all analyses were performed with a flow-rate of 1 ml/min at room temperature.

RESULTS AND DISCUSSION

Gel permeation chromatography on Sephadex G-25 with 0.1 M acetic acid

Chromatography of tetra- and hexasaccharide resulted in two unresolved peaks (Fig. 1). Poor resolution was obtained as further analysis with HPLC revealed. The hexasaccharide peak was already contaminated with 10% of tetrasaccharide at its peak maximum and the tetrasaccharide peak was contaminated too. In addition, oligosaccharide losses of 37.3% were caused by adsorption of tetra- and hexasaccharide on the stationary phase: the loss of tetrasaccharide was greater (Table I).

Gel permeation chromatography on Sephadex G-25 with 1.0 M sodium chloride as eluent

Separation of oligosaccharides can be significantly improved by using 1.0 *M* sodium chloride as eluent according to Flodin *et al.*[8], although the hexa- and tetrasaccharide peaks obtained still partly overlap, as confirmed in own experiments (Fig. 2). Adsorption effects were suppressed by using this eluent of higher ionic strength,

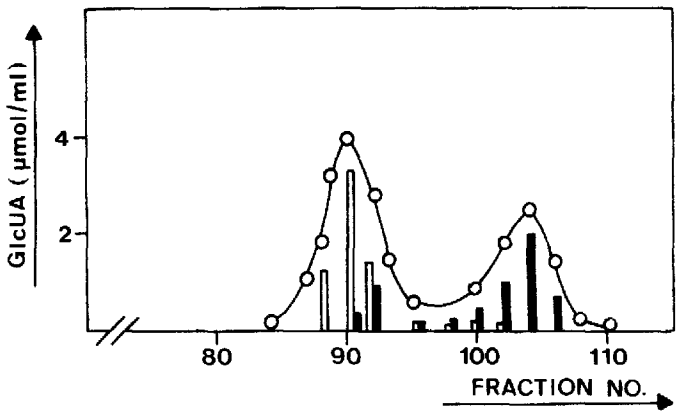

Fig. 1. Gel filtration chromatogram of 50 mg each of tetra- and hexasaccharides on Sephadex G-25 with 0.1 *M* acetic acid as eluent. Fractions of 2 ml were tested with the carbazole reaction for GlcUA-containing saccharides (○). The contents of tetra- (■) and hexasaccharide (□) were estimated by HPLC.

and losses of only 1% were detected (Table I). Flodin *et al.* employed only the GlcUA/GlcNAc ratio as a purity criterion. Moreover, use of this method means that peak contamination by other oligosaccharides cannot be determined. It is concluded that separation of odd-numbered HA oligosaccharides, *e.g.* two different trisaccharides, cannot be achieved by gel permeation chromatography because (1) they would elute between the already overlapping peaks and (2) gel permeation chromatography is based only on differences in molecular size and shape. Consequently, ion-exchange chromatography is required for separation of odd-numbered oligosaccharides. Furthermore, a second chromatography step on Sephadex G-10 is necessary in order to desalt oligosaccharide fractions obtained by gel permeation chromatography with 1.0 *M* sodium chloride as eluent.

Ion-exchange chromatography on Dowex 1-X8 (formate form)

Dowex resins differ in cross-linkage of polystyrene–quaternary ammonium salt and in size. HA oligosaccharides up to decasaccharide have been separated by stepwise[1,14] and linear gradient elution on different Dowex resins[12,13], but neither the

TABLE I

SEPARATION AND RECOVERIES OF HYALURONATE TETRA- AND HEXASACCHARIDES ON DIFFERENT GEL FILTRATION AND ION-EXCHANGE RESINS

Material	*Eluent*	*Tetra-saccharide*	*Hexa-saccharide*	*Recovery (%)*
Sephadex G-25	0.1 *M* Acetic acid	25.5 mg	37.2 mg	62.7
Sephadex G-25	1.0 *M* Sodium chloride	49.5 mg	49.5 mg	99.0
Dowex 1-X8 (formate form)	0–1.0 *M* Formic acid	35.2 mg	16.7 mg	51.9
DEAE Sephacel (chloride form)	0–0.1 *M* Sodium chloride	50.0 mg	49.5 mg	99.5
DEAE Trisacryl M (formate form)	0–1.0 *M* Formic acid	49.6 mg	48.0 mg	97.5
DEAE Sephacel (formate form)	0–1.0 *M* Formic acid	49.8 mg	49.4 mg	99.2
DEAE Trisacryl M (acetate form)	0–6.0 *M* Acetic acid	47.6 mg	42.5 mg	90.1
DEAE Sephacel (acetate form)	0–3.0 *M* Formic acid	49.7 mg	49.3 mg	99.0

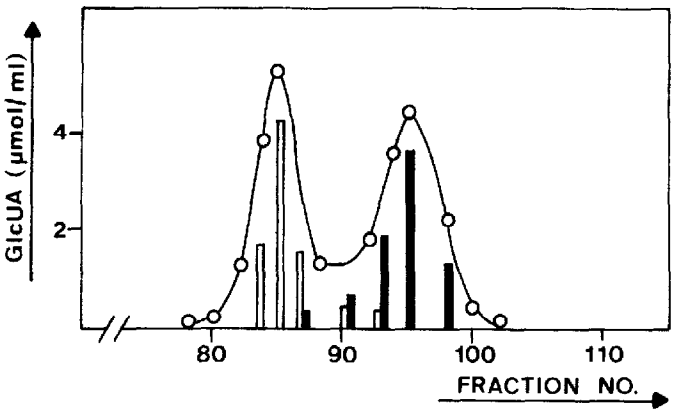

Fig. 2. Sephadex G-25 gel filtration chromatogram of 50 mg each of tetra- and hexasaccharides with 1 *M* sodium chloride as eluent. GlcUA-containing fractions (2 ml) were tested with the carbazole reaction (○) and the contents of tetra- (■) and hexasaccharides (□) were determined by HPLC.

separaton conditions nor the recoveries were reported. In own experiments a Dowex 1-X8 column (formate form), prepared according to Murphy *et al.*[19], was used. Fig. 3 shows a separation of tetra- and hexasaccharide achieved by increasing the ionic strength and the pH: 70.4% of the tetrasaccharide was desorbed selectively at 0.34 *M* formic acid and 33.4% of the hexasaccharide at 0.56 *M* formic acid. Higher losses of hexasaccharide were caused by the higher affinity of this saccharide for the ion-exchange material, resulting in increased adsorption. HPLC analysis showed only a little contamination of both peaks with the corresponding oligosaccharide.

Ion-exchange chromatography on DEAE Sephacel (chloride form)

Longas and Meyer[6] obtained a satisfactory separation of different trisaccharides and a tetrasaccharide employing DEAE cellulose chromatography material and a linear sodium chloride gradient. Tetra- and hexasaccharides were successfully separated without any contamination of the corresponding saccharide. Desorption properties for tetrasaccharide with 0.04 *M* sodium chloride and hexasaccharide with 0.08 *M* sodium chloride are remarkably low (Table II). Ion-exchange chromatography on DEAE Sephacel (chloride form) gives better yields of fractionated oligosaccharides, and a recovery of 99.5% was determined. As with gel permeation, tetra- and hexasaccharides have to be desalted by a second chromatographic step on Sephadex G-10.

Ion-exchange chromatography on DEAE Trisacryl M (formate form) and DEAE Sephacel (formate form)

Both ion-exchange methods resulted in a good resolution of oligosaccharides without any contamination as revealed by tests with HPLC. Recoveries were excellent at 97.6% and 99.2%, respectively. Table I shows the similar desorption data for tetra- and hexasaccharides obtained on DEAE Trisacryl M and DEAE Sephacel (both formate form). The use of DEAE Sephacel (formate form) showed a five times lower retention time than DEAE Trisacryl M material, which indicates swelling properties with increasing ionic strength that cause a decreased flow-rate.

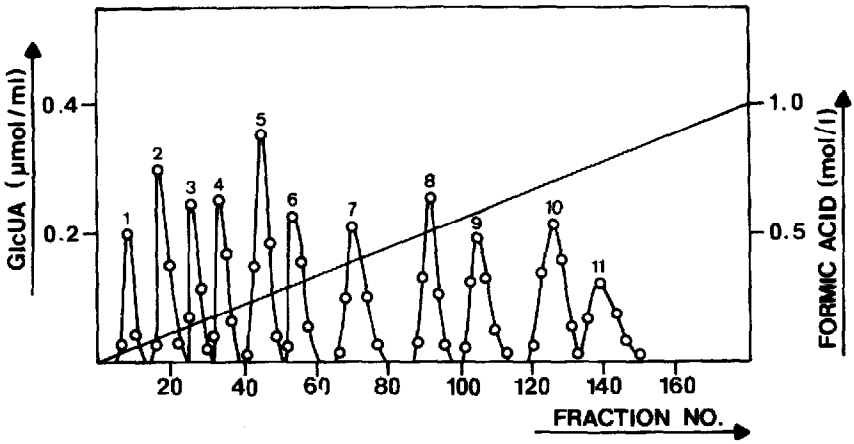

Fig. 3. Separation of even- and odd-numbered hyaluronate oligosaccharides on DEAE Sephacel (formate form). A mixture of purified oligosaccharides (4 mg of each saccharide) was applied to a modified DEAE Sephacel column and desorbed with a linear formic acid gradient (——). In 5.6-ml fractions, the saccharide content was estimated by the carbazole method (○). Peaks: 1 = trisaccharide; 2 = disaccharide; 3 = pentasaccharide; 4 = tetrasaccharide; 5 = trisaccharide*; 6 = heptasaccharide; 7 = hexasaccharide; 8 = pentasaccharide*; 9 = octasaccharide; 10 = heptasaccharide*; 11 = decasaccharide. Asterisks denote saccharides with terminal reducing GlcUA (see Table III).

Ion-exchange chromatography on DEAE Trisacryl M (acetate form) and DEAE Sephacel (acetate form)

Good resolutions and recoveries of tetra- and hexasaccharides were obtained in both experiments, and no contamination was detected by HPLC. In both cases, high acetic acid concentrations were needed for to desorb oligosaccharides from the resins (Table II). Tetrasaccharide was eluted with 0.8 *M* and hexasaccharide with 1.5 *M* acetic acid on DEAE Sephacel (acetate form), whereas DEAE Trisacryl M (acetate form) showed higher desorption data with 2.2 *M* and 3.9 *M* acetic acid, respectively (Table II). Adsorption losses could not be detected and excellent recoveries were achieved: 90.1% (DEAE Trisacryl M) and 99.0% (DEAE Sephacel). Swelling and agglutination of the resin material was observed in both cases; this increased with increasing ionic strength and pH. Because of the difficulty of evaporating high acetic

TABLE II

DESORPTION DATA OF HYALURONATE TETRA- AND HEXASACCHARIDES FROM DIFFERENT ION-EXCHANGE CHROMATOGRAPHY MATERIALS

Material	*Desorption data*	
	Tetrasaccharide	*Hexasaccharide*
Dowex 1-X8 (formate form)	0.34 *M* Formic acid	0.56 *M* Formic acid
DEAE Sephacel (chloride form)	0.04 *M* Sodium chloride	0.08 *M* Sodium chloride
DEAE Trisacryl M (formate form)	0.19 *M* Formic acid	0.35 *M* Formic acid
DEAE Sephacel (formate form)	0.17 *M* Formic acid	0.34 *M* Formic acid
DEAE Trisacryl M (acetate form)	2.20 *M* Acetic acid	3.90 *M* Acetic acid
DEAE Sephacel (acetate form)	0.80 *M* Acetic acid	1.50 *M* Acetic acid

TABLE III

DESORPTION DATA OF EVEN- AND ODD-NUMBERED HYALURONATE OLIGOSACCHARIDES OBTAINED FROM MODIFIED DOWEX 1-X8 AND DEAE SEPHACEL COLUMNS

A 4-mg amount of each hyaluronate oligosaccharide were separated on a column filled with Dowex 1-X8 (formate form) and DEAE Sephacel (formate form), respectively.

Saccharide	*Dowex 1-X8 (formate form) formic acid (M)*	*DEAE Sephacel (formate form) formic acid (M)*
Trisaccharide	0.08	0.05
Disaccharide	0.14	0.09
Glucuronic acid	0.21	0.12
Pentasaccharide	0.23	0.14
Tetrasaccharide	0.34	0.18
Tetrasaccharide*	0.34	0.18
Trisaccharide*	0.41	0.25
Heptasaccharide	0.46	0.30
Hexasaccharide	0.56	0.39
Hexasaccharide*	0.56	0.39
Pentasaccharide*	0.71	0.51
Octasaccharide	0.80	0.58
Octasaccharide*	0.80	0.58
Heptasaccharide*	0.92	0.70
Decasaccharide	1.00	0.76

acid concentrations, ion-exchange chromatography with acetic acid as eluent is insuitable for oligosaccharide separations.

Gel permeation chromatography is not advantageous for fractionation of odd-numbered oligosaccharides such as (β-GlcNAcl–4βGlcUAl–3GlcNAc) and (βGlcUAl–3βGlcNAcl–4GlcUA) because they hardly differ in molecular mass and size but differ in net charge. Ion-exchange chromatography exploits these charge differences and higher peak resolution of such oligosaccharides is obtained.

Of all the ion exchangers tested, the best separation effects and analysis times were obtained on DEAE Sephacel (formate form) and Dowex 1-X8 (formate form) for separating even- and odd-numbered oligosaccharides (Table III). Both modified Trisacryl M resins showed good resolution and recovery, but the analysis time was prolonged by swelling properties and a low flow-rate. DEAE Sephacel (formate form) is particularly suitable for separating a range of HA oligosaccharides because of high recoveries and resolution (Fig. 3), although it has much lower capacity than Dowex 1-X8 (formate form). Concentrations of oligosaccharides five times higher than those of pure saccharides can be applied to a Dowex 1-X8 (formate form) column in comparison with a DEAE Sephacel (formate form) column of equal dimensions, but Dowex 1-X8 resin shows poorer recovery.

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