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Sequential hydrolysis of hyaluronate by β-glucuronidase and β-*N*-acetylhexosaminidase

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1. Hyaluronate extracted from rooster comb was digested by a mixture of β-*N*-acetylhexosaminidase and β-glucuronidase with simultaneous dialysis for 96h. 2. The product, yielding 99.6% of a mixture of mono- and oligo-saccharides, was purified by gel chromatography and analysed for glucuronic acid, *N*-acetylglucosamine and other sugars. 3. The oligosaccharide portion was chromatographed on DEAE-cellulose, and the effluent fractions were analysed for glucuronic acid and *N*-acetylglucosamine, reduced with $NaBH_4$, digested by β-*N*-acetylhexosaminidase and subjected to acid hydrolysis and glucosamine determination. 4. GlcNAc-GlcA-GlcNAc, GlcA-GlcA-GlcNAc and GlcA-GlcA-GlcA-GlcNAc were the oligosaccharides obtained, which resulted from the transferase activity of the enzymes and represented 57% of the digestion products. The results demonstrate that this hyaluronate is an unbranched polymer of approximately equal amounts of glucuronic acid and *N*-acetylglucosamine. The data also indicate that if this glycosaminoglycan contains any of the neutral sugars for which it was analysed, their concentration must be <0.020% of the sum of the known components.

Hyaluronate, an acidic glycosaminoglycan isolated from a variety of animal tissues (Meyer, 1958; Jeanloz, 1970; Appel *et al.*, 1979), including rooster comb (Swann, 1968), is unique among this group of substances in that it does not appear to be heterogeneous. Since the early structural studies, it has been recognized that hyaluronate is composed of equimolar amounts of glucuronic acid and *N*-acetylglucosamine linked via β1→3 glucuronidic and β1→4 glucosaminidic bonds (Weissman & Meyer, 1953; Linker & Meyer, 1954; Hoffman & Meyer, 1957). The polymer was demonstrated to be a partly flexible and partly stiff (Darke *et al.*, 1975) chain of varying length (Balazs, 1958).

No differences were detected in the chemical composition of this glycosaminoglycan isolated from vitreous humour, synovial fluid, umbilical cord, skin, aorta, streptococci or a variety of mesenchymal tumours (Meyer, 1958). Nevertheless, other investigators have suggested that at least 10% of the sugar residues in hyaluronate from umbilical cord are not glucuronic acid or *N*-acetylglucosamine (Montgomery & Nag, 1963). Furthermore, arabinose and fucose have been reported in hyaluronate from bovine vitreous humour (Varma *et al.*, 1974).

In studies that suggested that hyaluronate was an unbranched chain, the initial enzymic cleavage was performed with endoglycosidases (Meyer, 1958). Thus, to obtain monosaccharides, this glycosaminoglycan was hydrolysed by hyaluronidase, and the products were digested by β-*N*-acetylhexosaminidase and β-glucuronidase (Linker *et al.*, 1955). The sequential cleavage of this polysaccharide by these exoglycosidases, which are specific for unsubstituted exo-β-*N*-acetylhexosaminidic and exo-β-glucuronidic linkages (Linker *et al.*, 1955; Levvy & Conchie, 1966), has not been reported, and it appeared to offer a better means to test for branching.

The present paper describes the sequential hydrolysis of hyaluronate of rooster comb by a mixture of highly purified β-*N*-acetylhexosaminidase and β-glucuronidase with simultaneous dialysis, purification by gel chromatography and chemical characterization of the digestion products, in an attempt to find variations of the polymer.

Materials and methods

Sodium hyaluronate of rooster comb (mol.wt. 1.57×10^6, as determined by viscometry; 0.166% protein; undetectable sulphate and *N*-acetyl-

galactosamine) was a gift from Dr. E. A. Balazs of this department.

β-*N*-Acetylhexosaminidase (EC 3.2.1.52) was isolated from bovine epididymis (Levvy & Conchie, 1966) and further purified by affinity chromatography on 2-acetamido-2-deoxy-D-glucono-1,4-lactone–Sepharose (Pokorny & Glaudemans, 1974). The specific activity of this preparation was 8000 *p*-nitrophenol units (1 unit = 1 μg of substrate transformed/h per mg of protein). β-Glucuronidase (EC 3.2.1.31) from bovine liver was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and purified on saccharo-1,4-lactone–Sepharose (Harris *et al.*, 1973). Its specific activity was 1440 phenolphthalein units (1 unit = 1 μg of substrate transformed/h per mg of protein). Anion exchange resin Durrum DA-X4 was a product of Durrum Chemical Corp., Palo Alto, CA, U.S.A. 2-Acetamido-2-deoxy-D-glucono-1,4-lactone was synthesized by the method of Levvy & Conchie (1966). Saccharo-1,4-lactone was from Calbiochem–Behring Corp., San Diego, CA, U.S.A.

Analytical methods

The hydrolytic activities of β-*N*-acetylhexosaminidase and β-glucuronidase were measured by the method of Levvy & Conchie (1966). They were also determined in 0.05 M-sodium acetate/0.05 M-sodium citrate/1 mM-EDTA, pH 4.3 (adjusted with acetic acid). Under these conditions, β-glucuronidase activity was 75% of that determined in the standard conditions, whereas the activity of β-*N*-acetylhexosaminidase was above 100%. *N*-Acetylglucosamine was determined by the method of Morgan & Elson (1934) as modified by Reissig *et al.* (1955). Glucuronic acid was quantified by a modification of Dische's (1947) procedure (Bitter & Muir, 1962) with glucuronolactone as the standard. Reducing hexose was assayed by the method of Park & Johnson (1949) with glucose as the standard. Glucosamine was determined by the Elson–Morgan reaction (1933). Neutral sugars were quantified by the orcinol method, after elution from a DA-X4 anion exchange column with sodium borate buffer, in a Technicon Autoanalyzer from Technicon Instruments Corp., Tarrytown, NY, U.S.A., connected to an Autolab System AA computing integrator from Spectra-Physics, Mountain View, CA, U.S.A. (Lee *et al.*, 1971; Spiro, 1972). Bovine albumin was the standard for protein assay (Lowry *et al.*, 1951).

Enzymic hydrolysis of hyaluronate

Unless otherwise indicated, the buffer used in this experiment was 0.05 M-sodium citrate/0.05 M-sodium acetate/1 mM-EDTA, pH 4.3 (adjusted with acetic acid). Digestion was carried out in 13 mm-internal-diameter dialysis tubing of 1000-mol.wt. cut off, to decrease enzyme inhibition by hydrolysis products. Hyaluronate (3.81 mg in 1.0 ml of buffer), β-glucuronidase (22000 units in 0.5 ml of 0.07 M-sodium acetate, pH 7.0) and β-*N*-acetylhexosaminidase (22000 units in 0.6 ml of 0.05 M-sodium citrate/0.05 M-sodium phosphate, pH 5.5) were mixed and dialysed against 1 litre of buffer at 4°C for 1 h. The dialysis tubing was transferred to a screw cap tube (15 cm × 1 cm) containing 15.0 ml of buffer and incubated under toluene at 37°C with constant shaking. All the reagents except the enzymes and the enzymes in buffer were used as separate controls. The diffusate was changed every 24 h and analysed for glucuronic acid and *N*-acetylglucosamine.

Gel filtration

Concentration of samples throughout these experiments was carried out *in vacuo* in a rotary evaporator at 37°C. The diffusates (24–48 h) from the enzymic hydrolysis of hyaluronate were combined, concentrated to one-third of the volume and desalted on a Bio-Gel P-2 (100–200 mesh) column (110 cm × 0.7 cm) equilibrated and eluted with water at a flow rate of 3.5 ml/h at room temperature. Fractions (1.8 ml) were collected and assayed for glucuronic acid and *N*-acetylhexosamine.

The non-diffusible materials from three identical digestions (approx. 7 μg of glucuronic acid each) at 96 h of reaction were combined, centrifuged to remove protein precipitates, concentrated to 0.5 ml, loaded on to a Bio-Gel P-2 column (30 cm × 0.4 cm) and eluted as described above. Carbohydrates in the eluting (0.27 ml) fractions were determined by the method of Park & Johnson (1949).

In a separate experiment, enzymic hydrolysis was stopped at 22 h by boiling for 10 min. A precipitate was removed by centrifugation, and the supernatant was shaken with an equal volume of chloroform. The carbohydrate-containing layer was chromatographed on a Bio-Gel P-300 (100–200 mesh) column (114 cm × 0.5 cm) equilibrated and eluted with 1 M-$MgCl_2$ at a flow rate of 2.5 ml/h at room temperature. Fractions (1.0 ml) were collected and analysed for protein and glucuronic acid.

Analytical gel-exclusion chromatography

The oligosaccharide pool from Bio-Gel P-2 was concentrated to 0.5 ml, and 1.3 mg (based on glucuronic acid) was chromatographed on a calibrated column of Sephadex G-25 (115 cm × 0.5 cm). Di- and tetra-saccharides, obtained by exhaustive digestion of the same hyaluronate with testicular hyaluronidase and glucuronic acid, were used in the calibration. The V_e for the trisaccharide was determined by extrapolation on a plot of elution volume versus log (molecular weight). The column was equilibrated and eluted with 1 M-NaCl at a flow

rate of 0.2ml/h at room temperature, and the collected fractions (0.3ml) were assayed for glucuronic acid and *N*-acetylglucosamine.

DEAE-Cellulose chromatography

The trisaccharide pool from Sephadex G-25 was desalted on Bio-Gel P-2, and 367μg (based on glucuronic acid) was applied to a column (1.2cm × 12cm) of DEAE-cellulose equilibrated with 0.03M-NaCl. Elution was accomplished with a linear gradient of 100ml of 0.03M-NaCl and 100ml of 0.1M-NaCl. Effluent fractions (1.2ml) were monitored for glucuronic acid as indicated.

T.l.c.

Preliminary analysis of the products of the enzymic hydrolysis of hyaluronate was performed on Silica Gel G for 2h or Silica Gel HR with the following solvent systems (by volume): A, n-butanol/acetic acid/diethyl ether/water (90:6:3:1) (Hay *et al.*, 1963); B, ethyl acetate/acetic acid/water (6:3:2); and C, 95% ethanol/pyridine/water (30:2:15). Carbohydrate components were located by the H_2SO_4 charring technique.

Transferase activity

In an attempt to find the origin of the high yield of oligosaccharides in the degradation products of hyaluronate, the transferase activities on the enzymes were tested in similar conditions. Using phenolphthalein β-glucuronide and *p*-nitrophenyl β-D-glucosaminide as the donor substrates, a modification of the method of Niemann & Buddecke (1979) was employed. Donor substrates (10μmol of each) were mixed with 22000 units of β-glucuronidase and 23000 units of β-*N*-acetylhexosaminidase in 1.5ml of 0.05M-sodium citrate/0.05M-sodium acetate/1mM-EDTA, pH4.3 (adjusted with acetic acid) and incubated under toluene at 37°C. Portions of the reaction mixture were removed at 24 and 48h and analysed by t.l.c. and calibrated gel chromatography. The chemical composition of the resulting oligosaccharides was also determined.

Reduction with $NaBH_4$

Samples were incubated with $NaBH_4$ in water at pH8.9 (adjusted with dilute NH_3). An excess of 400equiv. of $NaBH_4$ was used. Reaction was allowed at 27°C for 3h and stopped by lowering the pH of the reaction mixture to 4.0 with 0.1M-acetic acid. Excess reducing agent was removed by repeated distillation with an equal volume of dry methanol. Under these conditions, 100% of the free glucuronic acid used as control was reduced.

Results

Fig. 1 shows two carbohydrate pools from the enzymic hydrolysis of hyaluronate at 48h. They

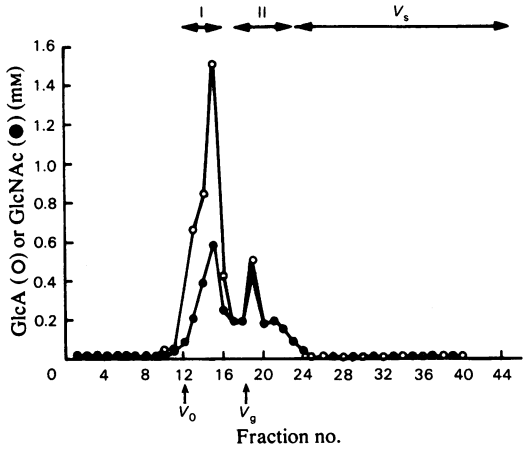


Fig. 1. *Elution profile of the combined (24–48h) diffusates of the enzymic hydrolysis of hyaluronate from Bio-Gel P-2*

Pool I contained a mixture of oligosaccharides, whereas pool II was composed of glucuronic acid and *N*-acetylglucosamine. V_0 was determined with Blue Dextran 2000; V_g is the glucose volume, and V_s is the salt volume. Chromatographic conditions are described in the text.

contained mono- and oligo-saccharides representing 95.5% of the degradation products (Table 1). These pools were spotted on Silica Gel G-coated t.l.c. plates and developed separately with solvents A and B. The first pool of Fig. 1 contained a carbohydrate with R_F of 0.1. Pool II displayed glucuronic acid, *N*-acetylglucosamine and a trace of disaccharide (Table 2). Further analysis of pool I (Fig. 1) on Silica Gel HR with solvent C revealed only one carbohydrate with a mobility of 0.85 (Table 2). Its chromatography on Sephadex G-25 with 1M-NaCl resulted in one carbohydrate peak that eluted at the V_e of the standard trisaccharide (Fig. 2). This pool was desalted on Bio-Gel P-2 and chromatographed on DEAE-cellulose with a linear gradient of NaCl. Its elution profile shows three oligosaccharide peaks (Fig. 3) whose molar glucuronic acid/*N*-acetylglucosamine ratios appear in Table 3. The R_F values of these peaks are listed in Table 2. Their actual yields at different hydrolysis times are shown in Scheme 1. Hydrolysis of these oligosaccharides in 4M-HCl (in de-aerated sealed tubes) for 15h followed by glucosamine determination indicated that the oligosaccharide eluted in pool I (Fig. 3) contained two glucosamine residues, whereas the others had only one (Table 3). Quantification of glucuronic acid before and after reduction with $NaBH_4$ demonstrated that this sugar was not in the reducing end of any of the oligosaccharides (Table 4). Digestion of pool I (Fig. 3) with β-*N*-acetylhexosaminidase

Table 1. *Sequential hydrolysis of hyaluronate by β-glucuronidase and β-N-acetylhexosaminidase*
Hyaluronate was incubated with the enzymes in dialysis tubing as described in the text. The diffusates were changed every 24 h and analysed for glucuronic acid and *N*-acetylglucosamine.

Time of diffusate collection (h)	GlcA (μmol)	GlcNAc (μmol)	GlcA/GlcNAc molar ratio	Yield (%)		GlcNAc‡ (%)
				Actual*	Theoretical†	
0	0	0	0	0	0	
24	7.34	5.98	1.23	68.00	75.00	
48	2.00	1.64	1.22	18.60	20.53	
72	0.31	0.18	1.72	2.50	3.15	
96	0.10			0.05	0.95	10.45

* Includes glucuronic acid and *N*-acetylglucosamine.

† Based on quantification of glucuronic acid only, and assuming a 1:1 molar ratio of glucuronic acid/*N*-acetylglucosamine in the starting material. This assumption is based on the glucuronic acid/glucosamine ratios obtained after acid hydrolysis of hyaluronate, as described in the text, in which 1.01:1 was the average from three different determinations; 100% yield = 9.78 × 2 = (μmol of GlcA in hyaluronate) 2.

‡ Detected only after acid hydrolysis and/or digestion of GlcNAc-GlcA-GlcNAc, pool I from DEAE-cellulose (Fig. 3 and Scheme 1) with *β-N*-acetylhexosaminidase (Table 3). This is the *N*-acetylglucosamine that occupied non-reducing positions and was not detectable by the method of Morgan & Elson (1934). It represents the difference between actual and theoretical yields.

Table 2. *T.l.c. of standards and degradation products of hyaluronate*
The products of the enzymic cleavage of hyaluronate at different stages of purification were spotted on Silica Gel G or HR and developed with the solvent systems described in the text.

Carbohydrate sample	Mobility in different solvents		
	A	B	C*
Arabinose	0.31	0.45	
N-Acetylgalactosamine	0.21	0.38	
Glucose		0.38	
N-Acetylglucosamine	0.21	0.48	
Glucuronic acid	0.06	0.21	
Mannose	0.28	0.41	
Ribose	0.36	0.50	
Rhamnose	0.44	0.61	
Standard disaccharide		0.17	0.94
Standard trisaccharide†		0.10	0.84
Standard tetrasaccharide		0.10	0.71
Bio-Gel P-2 (Fig. 1)			
Pool I		0.10	0.85
Pool II	0.06	0.21	
	0.21	0.48	
		0.17	
Sephadex G-25 (Fig. 2)			0.85
DEAE-cellulose (Fig. 3)			
Pool I			0.84
Pool II			0.80
Pool III			0.79
Bio-Gel P-2 (Fig. 4)			
Pool A		0.10	
		0.48	
Non-diffusible material from DEAE-cellulose (Figure not shown)		—	
Pool I			0.94
Pool II			0.84
Pool III			0.80

* Silica Gel HR plates were used, which were developed for 1 h.

† Determined after hydrolysis of the standard tetrasaccharide (30 μg based on glucuronic acid) by β-glucuronidase (36 units) in 0.1 M-sodium acetate, pH 4.5, at 37°C for 30 min.

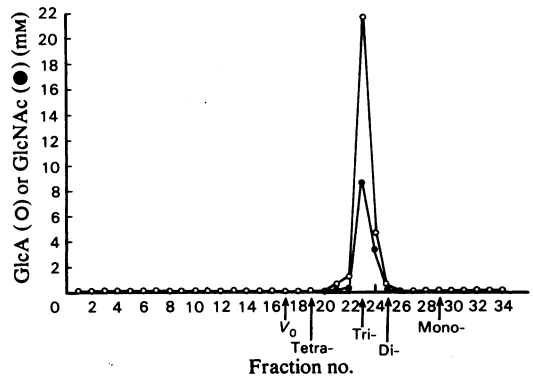


Fig. 2. *Sephadex G-25 chromatography of the oligosaccharide pool I of Fig. 1 on Bio-Gel P-2*
Mono-, Di-, Tri- and Tetra- are the positions of the standard mono-, di-, tri- and tetra-saccharides described in the text. V_0 was determined as indicated in the legend to Fig. 1.

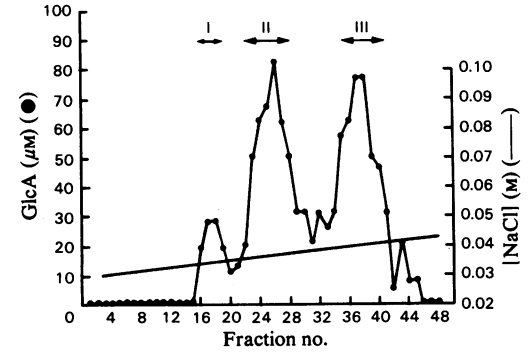


Fig. 3. *DEAE-cellulose chromatography of the trisaccharide peak from Sephadex G-25 (Fig. 2)*
GlcNAc-GlcA-GlcNAc, GlcA-GlcA-GlcNAc and GlcA-GlcA-GlcA-GlcNAc were eluted in pools I, II and III respectively. Conditions of chromatography are listed in the text.

yielded two reducing *N*-acetylglucosamine residues, whereas pools II and III showed no change in *N*-acetylglucosamine content (Table 3). The glucuronic acid/*N*-acetylglucosamine ratios of these pools before and after digestion by β-*N*-acetylhexosaminidase, glucuronic acid/glucosamine ratios after acid hydrolysis (Table 3) and the results of glucuronic acid determination before and after reduction with $NaBH_4$ (Table 4) indicate that the oligosaccharides eluted from DEAE-cellulose (Fig. 3) were: GlcNAc-GlcA-GlcNAc, GlcA-GlcA-GlcNAc and GlcA-GlcA-GlcA-GlcNAc in pools I, II and III respectively.

After 99.6% of the concentration of hyaluronate used in the enzymic hydrolysis had been accounted for in the diffusates (Table 1), the non-diffusible material was chromatographed on Bio-Gel P-2 (Fig. 4). Those fractions containing carbohydrate were pooled, monitored for glucuronic acid and *N*-acetylglucosamine and analysed by t.l.c. on Silica Gel G with solvent B. One carbohydrate spot with R_F of 0.1 and traces of *N*-acetylglucosamine were the only components detected (Table 2). These products were then applied to a DEAE-cellulose column (8 cm × 0.4 cm) and eluted under the conditions described in

Table 4. *Effect of reduction with $NaBH_4$ on the concentration of glucuronic acid in oligosaccharides from the enzymic hydrolysis of hyaluronate*
Portions of the pools from DEAE-cellulose (Fig. 3) were reduced with $NaBH_4$ as described in the text, and glucuronic acid was determined.

Carbohydrate-containing sample	GlcA* (nmol)	GlcA† (nmol)
Pool I (DEAE-cellulose)	69.1	69.5
Pool II	566.6	592.4
Pool III	273.0	276.10

* Determined before reduction with $NaBH_4$.
† Calculated after reduction with $NaBH_4$.

Table 3. *The molar ratios of glucuronic acid/N-acetylglucosamine in oligosaccharides from the enzymic degradation of hyaluronate*
The oligosaccharide peak obtained from Sephadex G-25 (Fig. 2) was chromatographed on DEAE-cellulose (Fig. 3). The eluted pools were assayed for glucuronic acid and *N*-acetylglucosamine before and after digestion by β-*N*-acetylhexosaminidase. Portions of these pools were hydrolysed in acid as described in the text, and glucuronic acid/glucosamine ratios were determined.

Oligosaccharide-containing sample	GlcA/GlcNAc* (molar ratio)	GlcA/GlcNAc† (molar ratio)	GlcA/GlcN‡ (molar ratio)
Pool I (DEAE-cellulose)	1.03	0.50	0.49
Pool II	2.04	2.03	2.01
Pool III	2.97	2.98	2.96

* Determined by the method of Morgan & Elson (1934) before digestion by β-*N*-acetylhexosaminidase.
† Quantified by the method of Morgan & Elson (1934) after hydrolysis by β-*N*-acetylhexosaminidase (Levvy & Conchie, 1966).
‡ Determined by the method of Elson & Morgan (1933) after acid hydrolysis.

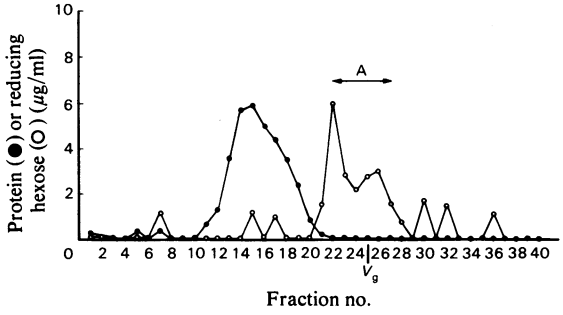
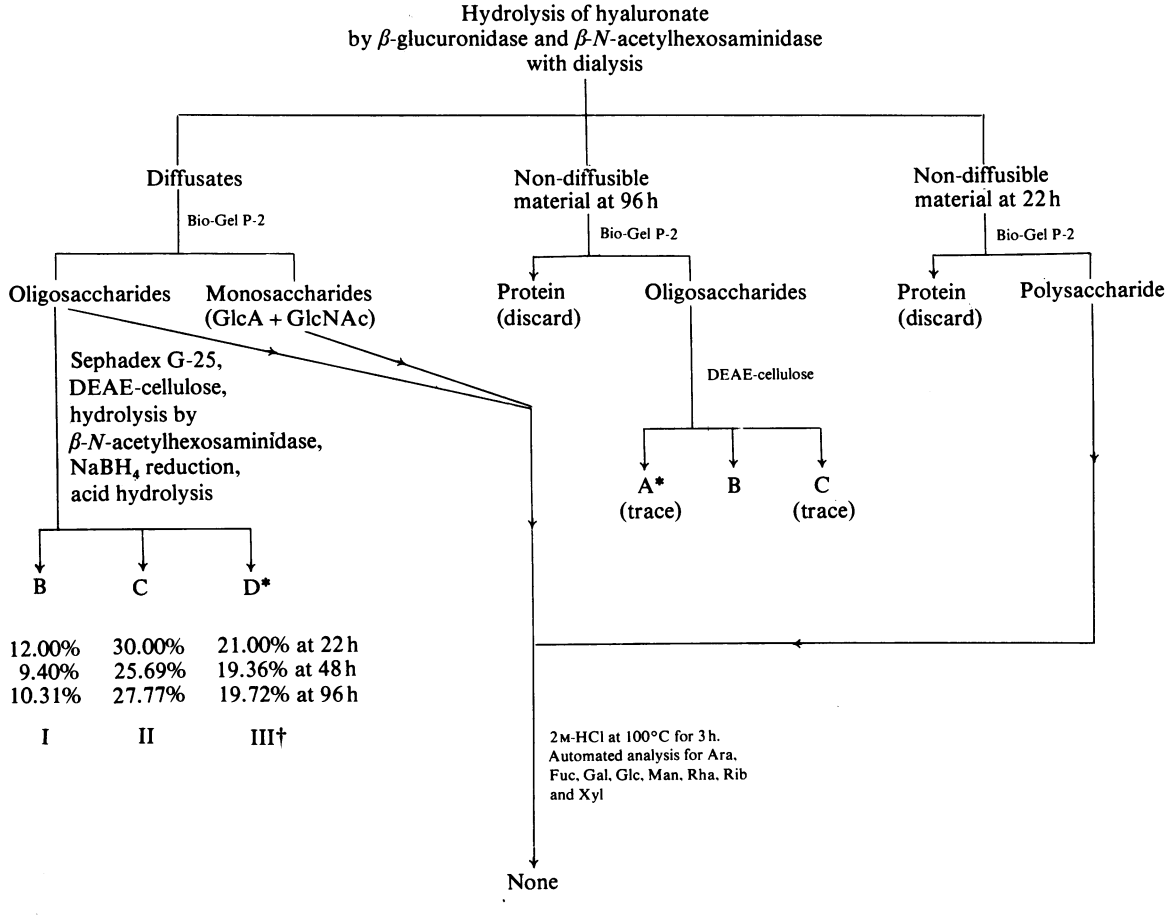


Fig. 4. *Elution pattern of the non-diffusible material of the enzymic degradation of hyaluronate from a Bio-Gel P-2 column (30 cm × 0.4 cm)*
A indicates the carbohydrate pool; V_g, glucose volume. Data are representative of three identical experiments. See the text for further details.

the previous section. Their elution profile displayed: (*a*) a fast eluting peak that contained 1 mol of glucuronic acid/mol of *N*-acetylglucosamine and had the mobility of the standard disaccharide on Silica Gel HR (Table 2); (*b*) GlcNAc-GlcA-GlcNAc; and (*c*) traces of GlcA-GlcA-GlcNAc (Scheme 1).

Since the concentration of carbohydrates obtained from the non-diffusible material was too low for further quantitative analysis, enzymic cleavage was interrupted when only 69% of the polysaccharide had been degraded. Fig. 5 shows that the undigested portion of hyaluronate was eluted in the void volume of a Bio-Gel P-300 column. This result demonstrates that the undegraded polysaccharide had a relatively high molecular weight, and suggests that hyaluronidase was not present in the reaction



* A, GlcA-GlcNAc, early eluting pool from DEAE-cellulose column (0.4 cm × 8 cm) (results not shown); B, GlcNAc-GlcA-GlcNAc; C, GlcA-GlcA-GlcNAc; D, GlcA-GlcA-GlcA-GlcNAc.
† Corresponding pools from DEAE-cellulose (Fig. 3).

Scheme 1. *Sequential enzymic hydrolysis of hyaluronate*
Abbreviation used: Rha, rhamnose.

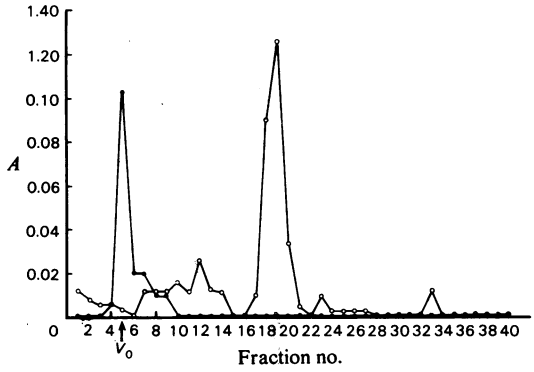


Fig. 5. *Purification of the undigested portion of hyaluronate at 22 h of enzymic hydrolysis on Bio-Gel P-300*
A constant pressure of 20 cm of water was maintained throughout the experiment. V_0 was determined as indicated in the legend to Fig. 1. ●, A_{530} after carbazole assay for glucuronic acid; O, A_{280}.

mixture. Hyaluronidase activity in the preparations of β-glucuronidase and β-*N*-acetylhexosaminidase used in the present study was also tested by the turbidimetric method of Tolksdorf *et al.* (1949) and was not detected.

Neutral sugars

Preliminary t.l.c. of the degradation products of hyaluronate after hydrolysis in 2 M-HCl at 100°C indicated that 3 h were required for complete cleavage of the oligo- and poly-saccharides. Therefore, all the digestion products were hydrolysed under these conditions. A sample containing a known concentration of arabinose, and another one containing glucuronic acid and *N*-acetylglucosamine were used as controls. The hydrolysates were analysed for arabinose, fucose, galactose, glucose, mannose, rhamnose, ribose and xylose as described in the Materials and methods section. The concentrations used, based on the contents of glucuronic acid and *N*-acetylglucosamine, were: 2840 nmol from 95.5% of the digestion products (Table 1) and 1560 nmol from the undigested reducing end (Fig. 5), which represented 31% of the starting concentration of hyaluronate. Although the system could detect 0.4 nmol of neutral sugar, none of the sugars listed above were found. Scheme 1 shows an outline of the sequential enzymic cleavage of hyaluronate, purification and characterization of the hydrolysis products.

The glucuronate and *N*-acetylglucosamine residues from 1:1 molar ratios of phenolphthalein β-glucuronide and *p*-nitrophenyl β-D-glucosaminide were converted into a mixture of GlcA-GlcNAc (5.7%), GlcA-GlcA-GlcNAc (14.8%), GlcA-GlcA-GlcA-GlcNAc (9.8%) and GlcNAc-GlcA-GlcNAc (1.0%).

Discussion

The hydrolytic activities of β-glucuronidase and β-*N*-acetylhexosaminidase are characterized by the cleavage of unsubstituted exo-β-glucuronidic and exo-β-*N*-acetylhexosaminidic linkages (Linker *et al.*, 1955; Levvy & Conchie, 1966). This leads to the expectation that the sequential cleavage of hyaluronate with these enzymes will yield glucuronic acid and *N*-acetylglucosamine.

In the sequential enzymic hydrolysis of hyaluronate described in the present paper, 57% of the degradation products were oligosaccharides of three and four sugar residues (Scheme 1). The origin of these oligosaccharides was assessed by the results of the assay for transferase activity of β-glucuronidase and β-*N*-acetylhexosaminidase with synthetic donor substrates; 31% of the glucuronate and *N*-acetylglucosamine residues from 1:1 molar ratios of phenolphthalein β-glucuronide and *p*-nitrophenyl β-D-glucosaminide under the same conditions was converted into a mixture of di-, tri- and tetrasaccharides (see the Results section).

The chemical composition of the above compounds agrees with that of the oligosaccharides obtained from the enzymic hydrolysis of hyaluronate (Scheme 1). The high transferase activity of β-glucuronidase was demonstrated by their content of glucuronate residues. These results present evidence for the high acceptor capacity of glucuronyl moieties in the conditions of the present work, and indicate that oligosaccharides of such chemical composition were indeed derived from transglycosylation.

It may be concluded from these observations that the oligosaccharides resulting from the enzymic degradation of hyaluronate described herein were not preformed in the polymer but were products of transglycosylation. In support of this conclusion is the high-molecular-weight polysaccharide obtained at 22 h of reaction (Fig. 5), which in the presence of a trace of testicular hyaluronidase, an endo-*N*-acetylhexosaminidase, was not produced (K. Meyer & C. Bergmann, unpublished work).

The degree of transferase activity could not be established; but it appears from the percentage of oligosaccharides obtained at different reaction times (Scheme 1) that hydrolysis and transglycosylation were taking place concomitantly. Only traces of glucuronido-*N*-acetylglucosamine were detected in the reaction products (Table 2 and Scheme 1). This suggests that its acceptor capacity was higher than its diffusion rate through the dialysis membrane.

The glucuronic acid/*N*-acetylglucosamine ratio in the hyaluronate used in the present study was

approx. 1 (Table 1). The lower yield of *N*-acetylglucosamine (10.45%) in the diffusates (Table 1) was due to the amount of this residue that occupied non-reducing positions and was undetectable by the method used in the quantification (Morgan & Elson, 1934). This difference, however, was accounted for by the 10.3% yield of GlcNAc-GlcA-GlcNAc at 96h of reaction (Scheme 1).

No neutral sugar components were found in the degradation products of hyaluronate, even though the system used in the analysis could detect 0.4nmol. Furthermore, the data herein indicate that if hyaluronate of rooster comb contains any of the neutral sugars for which it was analysed (Scheme 1), their concentration must be <0.020% of the sum of the known components.

The results of this investigation present further evidence in support of the chemical composition of hyaluronate. Thus hyaluronate of rooster comb has the same chemical composition as the hyaluronates from a variety of sources including umbilical cord and bovine vitreous humour, which were analysed previously (Meyer, 1958).

The enzymic cleavage of hyaluronate proceeded without interruption. This implies that the polymer is an unbranched chain of glucuronic acid and *N*-acetylglucosamine as suggested previously (Meyer, 1958).

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References

Appel, A., Horwitz, A. L. & Dorman, A. (1979) *J. Biol. Chem.* **255**, 12199–12203

Balazs, E. A. (1958) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **17**, 1086–1093

Bitter, T. & Muir, J. (1962) *Anal. Biochem.* **4**, 330–334

Darke, A., Finer, E. A., Moorhouse, R. & Rees, A. D. (1975) *J. Mol. Biol.* **99**, 477–486

Dische, Z. (1947) *J. Biol. Chem.* **167**, 189–198

Elson, L. A. & Morgan, W. T. J. (1933) *Biochem. J.* **27**, 1824–1828

Harris, R. G., Rowe, J. J. M., Stewart, P. S. & Williams, D. C. (1973) *FEBS Lett.* **29**, 189–192

Hay, G. W., Lewis, B. A. & Smith, F. (1963) *J. Chromatogr.* **11**, 479–486

Hoffman, P. & Meyer, K. (1957) *Harvey Lect.* **51**, 88

Jeanloz, R. W. (1970) in *The Carbohydrates* (Pigman, W., Horton, D. & Herp, A., eds.), vol. IIB, pp. 589–621, Academic Press, New York

Lee, Y. C., Johnson, G. S., White, B. & Scocca, J. (1971) *Anal. Biochem.* **43**, 640–643

Levvy, G. A. & Conchie, J. (1966) *Methods Enzymol.* **8**, 571–581

Linker, A. & Meyer, K. (1954) *Nature (London)* **174**, 1192–1194

Linker, A., Meyer, K. & Weissmann, B. (1955) *J. Biol. Chem.* **213**, 237–248

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Meyer, K. (1958) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **17**, 1075–1077

Montgomery, E. & Nag, S. (1963) *Biochim. Biophys. Acta* **74**, 300–302

Morgan, W. T. J. & Elson, L. A. (1934) *Biochem. J.* **28**, 988–995

Niemann, R. & Buddecke, E. (1979) *Biochim. Biophys. Acta* **567**, 196–206

Park, J. T. & Johnson, M. J. (1949) *J. Biol. Chem.* **181**, 149–151

Pokorny, M. & Glaudemans, C. P. J. (1974) *FEBS Lett.* **50**, 66–69

Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955) *J. Biol. Chem.* **217**, 959–966

Spiro, R. G. (1972) *Enzymes* **28**, 3–48

Swann, D. A. (1968) *Biochim. Biophys. Acta* **160**, 96–105

Tolksdorf, S., McCready, M. H., McCullagh, D. R. & Schwenk, E. (1949) *J. Lab. Clin. Med.* **34**, 74–89

Varma, B., Varma, R. S., Allen, W. S. & Wardi, A. (1974) *Biochim. Biophys. Acta* **263**, 584–588

Weissman, B. & Meyer, K. (1953) *J. Am. Chem. Soc.* **76**, 1753–1757