Fractionation of heparin-derived oligosaccharides by gradient polyacrylamide-gel electrophoresis

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Heparin-derived oligosaccharides, prepared by using flavobacterial heparinase, having a high degree of heterogeneity (sequence variability) were resolved into sharp well-defined bands by using polyacrylamide gel electrophoresis (PAGE). The use of a stacking gel and a high-density-pore-gradient resolving gel was primarily responsible for the success of this separation. Low- Mn standards of known structure and having a degree of polymerization (dp) 2-6 were used to establish that the separation on gradient PAGE was primarily dependent on molecular size. High-Mn oligosaccharides (dp 8-20) were prepared using strong-anion-exchange h.p.l.c. and were used to help characterize the gradient PAGE separation. Kinetic profiles were obtained for the depolymerization of heparin and heparan sulphate with heparinase and heparitinase respectively. The utility of this approach in sequencing oligosaccharides derived from glycosaminoglycans is discussed.

INTRODUCTION
The sequencing of complex polysaccharides such as GAGs is a difficult problem. This is because GAG preparations contain polysaccharide chains of different lengths (polysaparity), and chains of defined length may have different primary structure or sequences (heterogeneity). Short sequences, such as disaccharides, tetrasaccharides and hexasaccharides, have been prepared by enzymic depolymerization of the heparin polymer and characterized, after chromatographic purification, by rigorous structural proof (Linker & Hovingh, 1984; Merchant et al., 1985; Linhardt et al., 1986a). However, characterization of longer sequences of heparin requires improved separation methods.

Dermatan sulphate, chondroitin sulphate and hyaluronic acid GAGs have been sized and banded on polyacrylamide gels (Min & Cowman, 1986). Partial enzymic cleavage of these GAGs results in the formation of high-Mn oligosaccharides having common repeating sequences (Knudsen et al., 1984; Hampson & Gallagher, 1984; Cowman et al., 1984). By staggering the loading of samples on to the polyacrylamide gel, a single gel has been used to resolve many of the oligosaccharides comprising these GAGs (Hampson & Gallagher, 1984). However, this approach fails to band the oligosaccharides derived from the partial depolymerization of heparin and heparan sulphate GAGs.

We report here that partial enzymic cleavage of heparin and heparan sulphate results in high- and low-Mn oligosaccharides that resolve into bands with a single loading by utilization of a stacking gel and a high-density pore-gradient resolving gel. Characterization of the gel with h.p.l.c.-purified heparin-derived oligosaccharides indicates that separation has occurred primarily according to size (from dp 2 to greater than dp 20) and that structurally diverse oligosaccharides of a given dp were also resolved. The kinetic profile of the enzymic depolymerization of heparin and heparan sulphate is also described.

EXPERIMENTAL
Materials
Heparin sodium salt, from porcine intestinal mucosa (145 units/mg), was obtained from Hepar Industries, Franklin, OH, U.S.A. Heparan sulphate, from bovine kidney, and chondroitin 4- and 6-sulphates were from Sigma Chemical Co., St. Louis, MO, U.S.A. Heparinase (EC 4.2.2.7) was purified from Flavobacterium heparinum [5 units (μmol/min)/mg] (Yang et al., 1985; Linhardt et al., 1984), and heparinase (1.5 units/mg) was also obtained from Sigma. Heparitinase (EC 4.2.2.8; 1 unit/mg) and chondroitinase ABC (EC 4.2.2.4; 1 unit/mg) were obtained from Miles Laboratories, Elkhart, IN, U.S.A. Electrophoresis was performed on a Hoefer SE600 vertical-slab-gel unit from Hoefer Scientific Instruments, San Francisco, CA, U.S.A. The power source was supplied by a Bio-Rad (Richmond, CA, U.S.A.) model 1420B power source. Gel scans were performed with an E.C. 910 scanning densitometer with 0.3 mm × 3.0 mm slit width and 480-650 nm range, from E.C. Apparatus, St. Petersburg, FL, U.S.A. Gradients were prepared with a 250 ml gradient-elution apparatus from Kontes, Evanston, IL, U.S.A. U.V. spectroscopy was performed on a Shimadzu model UV-160 spectrophotometer.

S.a.x.h.p.l.c. was performed on a Spherisorb (5 μm particle size) column of dimensions 4.6 mm × 25 cm, with a 4.6 mm × 5 cm guard column from Phase Separations,

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; dp, degree of polymerization (i.e. for a disaccharide, dp = 2 etc.); GAG, glycosaminoglycan; s.a.x. strong-anion-exchange; g.p.c., gel-permeation chromatography; TEMED, NNN'-tetramethylethylenediamine; AUFs, absorbance units full scale; l.p.l.c., low-pressure liquid chromatography; Idu, iduronic acid; ΔIdu, 4-deoxy-a-L-threo-hex-4-enopyranosyluronic acid; GlcA, glucuronic acid; GlcN, glucosamine; S, sulphate; Ac, acetate.

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Norwalk, CT, U.S.A. G.p.c.–h.p.l.c. utilized a 7.5 mm x 40 cm TSK G2000SW gel-permeation column from Phenomenex, Palos Verdes, CA, U.S.A. Sephadex G-50 (superfine grade) was from Pharmacia Biochemicals, Piscataway, NJ, U.S.A. Spectraflow dialysis tubing (M, cut-off 1000) was purchased from Spectrum Medical, Los Angeles, CA, U.S.A. Acrylamide (ultrapure), Tris, Alcian Blue 8GX, Bromophenol Blue and ammonium persulphate were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Glycerine hydrochloride, disodium EDTA, boric acid, Azure A, N,N′-methylenebisacrylamide and TEMED were from Fisher Chemical Company, Fair Lawn, NJ, U.S.A. All chemicals utilized were of reagent grade.

Methods

Enzymic depolymerization of GAGs. To 1 ml of a solution containing heparin (8 mg) in 0.25 M-sodium acetate/2.5 mM-calcium acetate, pH 7.0, 50 μl containing 5 m.i.u. of purified heparinase or commercial heparinase was added and incubated at 30 °C. The reaction was monitored by removal of 10 μl aliquots, which were then added to 990 μl of 0.03 M-HCl, and absorbance was monitored at 232 nm. The reaction was complete within 24 h as determined by a constant absorbance. The addition of more enzyme caused no observable increase in absorbance. During the course of reaction, aliquots were removed, heated to 100 °C for 10 min to inactivate the enzyme and then were stored frozen. The absorbance measured at 232 nm at the time of aliquot removal was divided by the absorbance measured at the time of reaction completion and multiplied by 100 to calculate the percentage of reaction completion.

Heparan sulphate (375 μl of an 8 mg/ml solution in 0.25 M-sodium acetate/2.5 mM-calcium acetate, pH 7.0) was treated with 10 m.i.u. of heparitinase in an analogous fashion to the heparin reaction, except that the reaction was performed at 43 °C and was complete within 4 h. The addition of more heparitinase resulted in no further increase in absorbance. A mixture of chondroitin 4- and 6-sulphates was depolymerized using chondroitinase ABC by the method of Cowman et al. (1984).

Preparation of polyacrylamide gels. The resolving gel and lower buffer chamber contained 0.1 M-boric acid/0.1 M-Tris/0.01 M-disodium EDTA buffer, pH 8.3. The stacking gel was prepared in the same buffer, adjusted to a pH of 6.3 with concentrated HCl. The upper buffer chamber contained 0.2 M-Tris/1.25 M-glycine hydrochloride, pH 8.3. Solutions were prepared containing 11.52% (w/v) acrylamide with 0.48% (w/v) NN-bisacrylamide and 1% (w/v) sucrose in resolving-gel buffer, and 23% (w/v) acrylamide with 2% (w/v) NN-bisacrylamide and 15% (w/v) sucrose in resolving-gel buffer.

Gels were poured vertically, using a gel-pouring stand (Hoffer), between glass plates (16 cm × 32 cm) separated by 1.5 mm spacers. Gradient gels were poured by adding 35 ml of 12% total acrylamide (acrylamide + bisacrylamide) solution to the front chamber of the gradient apparatus, which was continuously mixed by a magnetic stirrer, and 35 ml of 25% total acrylamide solution to the back chamber. The solutions in the front chamber and back chambers were separated by a short length of clamped tubing. Ammonium persulphate, 200 μl of a 10% (w/v) solution in water, was added to the front chamber and 100 μl to the back chamber, followed by addition of 15 μl of TEMED to both the front and the back chamber. The clamp between the chambers was removed and the acrylamide solution, in the front chamber, passed by gravity feed into two metal tubes inserted between the glass plates along the spacers to the bottom of the gel sandwich. The gel was poured from the bottom up by using a pressure head of 1 m with a flow rate of 10 ml/min delivering 68 ml of gradient. The metal tubes were then carefully removed so as not to disturb the gradient. Butanol saturated with water (0.5 ml) was layered across the top of the gel and polymerization, which began at the top of the gel, was completed within 20 min. Non-gradient gels were prepared with and without stacking gels by the method of Hampson & Gallagher, 1984.

Electrophoresis of heparin- and h.c.paran sulphate-derived oligosaccharides

Before electrophoresis, the butanol layer was removed, and the top of the resolving gel was washed with water and then resolving-gel buffer. A 10 ml portion of stacking-gel solution containing 4.75% (w/v) acrylamide with 0.25% (w/v) of NN-bisacrylamide in stacking gel buffer, 200 μl of 10% (w/v) ammonium persulphate and 15 μl of TEMED was applied to the top of the resolving gel and a comb (15 × 5 mm well formers) was inserted. After polymerization (30 min) the comb was removed and the wells were rinsed with stacking-gel buffer.

The upper buffer chamber of the electrophoresis apparatus was filled with 0.2 M-Tris/1.25 M-glycine. Oligosaccharide samples (20 μl) were combined with 20 μl of 50% (w/v) sucrose in stacking-gel buffer placed in the bottom of the wells, using a microsyringe. To an empty well, 50 μl of 0.1% (w/v) solution of Bromophenol Blue, prepared in the 50% (w/v) sucrose stacking-gel buffer solutions was added.

Electrophoresis was performed on two gels at 500 V and at 80 mA for 6 h, at which time the Bromophenol Blue marker had migrated approx. 18 cm into the resolving gel. Gels were removed from the glass plates and stained for 30 min either in Alcian Blue 0.5% (w/v) in 2% (v/v) acetic acid or in 0.08% (w/v) Azure A in water. Destaining was accomplished with successive washes of 2% (v/v) acetic acid for Alcian Blue-stained gels or with water for Azure A-stained gels. All gels shown were stained with both dyes.

S.a.x.h.p.l.c. and g.p.c.–l.p.l.c. preparation of heparin-derived oligosaccharide Mf. standards. S.a.x.h.p.l.c. was performed as described previously (Rice et al., 1985). Briefly, the column was equilibrated with 0.2 M-NaCl pH 3.5. Heparin oligosaccharides (4 mg in 0.5 ml) produced in a 30% complete heparinase reaction were applied to the column using a 1 ml fixed-volume loop. The column was eluted with a linear gradient [concentration (y, in m) at any time (x, in s) = 0.0001 x + 0.2] of NaCl at pH 3.5 with a flow rate of 1.5 ml/min. The separation was monitored by u.v. absorbance at 232 nm (0.02 AUFS). Fractions were collected (1 ml), desalted by dialysis in 1000-Mf. cut-off bags and concentrated by freeze-drying. Individual fractions were re-applied to the s.a.x.h.p.l.c. column, and pure fractions were collected, desalted by dialysis and quantified by u.v. absorbance at 232 nm (Linker & Hovingh, 1972).
Electrophoretic fractionation of heparin-derived oligosaccharides

Low-\(M_r\) standards were size-separated by applying 8 mg of depolymerized heparin (100% reaction completion) to a low-pressure Sephadex G-50 (superfine grade) g.p.c. column (1.5 cm × 240 cm) and eluting with 0.2 M-NaCl at a flow rate of 10 ml/h. Fractions corresponding to disaccharide, tetrasaccharides, heparitinase and heparan sulphate

G.p.c.–h.p.l.c. analysis of heparin oligosaccharide \(M_r\) standards

Purified oligosaccharides collected from s.a.x.h.p.l.c. corresponding to 0.05 absorbance units at 232 nm were applied to the TSK-G2000SW column in a 100 \(\mu\)l fixed-volume loop. The column was eluted at 1 ml/min with 1.5 M-NaCl, pH 3.5, and peaks were detected at 232 nm with 0.02 AUFS. Retention times were measured by an Apple IIe microcomputer equipped with an A/D interface and driven by Chromatograph Software (Interactive Software, State College, PA, U.S.A.). The void and total volumes were measured with dilute solutions of Blue Dextran and NaN\(_5\).

**RESULTS**

Enzymic cleavage of both heparin by heparinase and heparan sulphate by heparitinase yields a final repeatable mixture of oligosaccharides of sizes ranging from disaccharide to oligosaccharides larger than decasaccharides (Fig. 1). These oligosaccharides contain an unsaturated non-reducing end sugar which has a chromophore, \(e_{520} = 5.2 \times 10^9\) M\(^{-1}\) cm\(^{-1}\) (Linker & Hovingh, 1972).

Electrophoresis of these oligosaccharides on a conventional isocratic gel used by previous workers (Hampson & Gallagher, 1984; Cowman et al., 1984) failed to result in their banding. The addition of a stacking gel on top of the 10\%-(w/v)-acrylamide isocratic resolving gel provided a marked increase in resolution, resulting in banding. However, in this system only high-\(M_r\) oligosaccharides (dp > 10) were resolved. Lower-\(M_r\) oligosaccharides (dp 4–10) were resolved better on a 20\%-(w/v)-acrylamide isocratic gel (with a stacking gel); however, this in turn reduced the sharpness of the banding obtained for the higher-\(M_r\) oligosaccharides. By the use of a 12–25\% (w/v) (total acrylamide) gradient, a broad range of \(M_r\) species could be resolved into sharp bands. By simultaneously casting a gradient of cross-linker (Margolis & Wrigley, 1975) of 0.48–2\% (w/v), the average pore size could be controlled to resolve even the smallest oligosaccharides (dp 2–4). The buffer used in the resolving gel was identical with that used in nucleic acid sequencing (Maxam & Gilbert, 1977). By increasing the concentration of stacking-gel buffer the electrophoresis time could be reduced without effecting the resolution.

Gradient gels, cast at different times, gave reproducible separations. Several gradient resolving gels could be cast and stored refrigerated for up to a week without change in resolving capacity. The linearity of the gradient resolving gel was measured by addition of Azure A dye (1 ml of 0.08\% (w/v)) to the back gradient chamber before pouring the gel (Margolis, 1969). Densitometer scanning across the entire length of the resulting gel

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showed a linear change in dye concentration with a correlation coefficient (r) of 0.997.

Electrophoresis was performed at 250, 500 and 1000 V. Improved resolution of the oligosaccharides was observed at higher voltages; however, at 1000 V, appreciable heating of the gel occurred, requiring the apparatus to be cooled, and resulting in ‘smiling’ (i.e. concave) bands. Two gels were typically run in a cooled apparatus (water recirculating at 20 °C) at 500 V for 6 h, at which time Bromophenol Blue had migrated approx. 18 cm into the resolving gel.

Sample volumes of 40 μl containing between 10 and 40 μg of a single component could be used without loss of band sharpness. The presence of salts, either NaCl or sodium acetate, at concentrations over 0.3 M interfered with stacking and resulted in a reduction of resolution and band sharpness. Gradient preparative gel electrophoresis under the same conditions used a 32 cm × 16 cm × 3 mm gel with sample load of up to 30 mg dissolved in 2 ml also resulted in sharp, well-resolved, bands.

Gels could be stained (Whiteman, 1973) to reveal bands using either Azure A or Alcian Blue dye solutions. Both dyes stained heparin- and heparan sulphate-derived oligosaccharides, but gave the greatest sensitivity when staining gels containing heparin-derived oligosaccharides. Azure A staining resulted in a 2-fold increase in sensitivity (100 μg of depolymerized heparin) over Alcian Blue staining as determined by a minimum level of visual band detection. Alcian Blue stained all the oligosaccharides obtained on the depolymerization of heparin, whereas Azure A failed to stain the disaccharide. Sensitivity could be increased and the disaccharide I (see structure given below) revealed by using a dual staining method of first staining with Alcian Blue, followed by Azure A staining and destaining. Fragments that contain less than three sulphate groups, obtained on depolymerization of heparan sulphate, might not be visible by this method of staining (Cowman et al., 1984).

Size purification of heparin-derived oligosaccharides by g.p.c.–l.p.l.c. on a Sephadex G-50 (superfine grade) gel-permeation column provided size-uniform oligosaccharide mixtures (Fig. 2). These sized oligosaccharides mixtures were resolved into multiple components when applied to the gradient gel (Fig. 3). The electrophoresis of the purified and structurally defined heparin-derived disaccharide I, tetrasaccharides (II, III and IV) and hexasaccharide (V) were conducted individually in adjacent lanes to determine their order of migration (results not shown). In the depolymerization mixture, disaccharide I, ΔIdu2S(1→4)-α-D-GlcNS6S, migrated furthest and could be resolved from the tetrasaccharides only when Bromophenol Blue was run a full 18 cm into the resolving gel (Fig. 3). The tetrasaccharide mixture obtained from g.p.c.–l.p.l.c. was composed of three major tetrasaccharides having different sequences. Isolation by s.a.x.h.p.l.c. and characterization of these sequences by 1H and 13C n.m.r. confirmed that two of the compounds were, as previously described, tetrasaccharides: ΔIdu2S(1→4)-α-D-GlcNS6S(1→4)-α-L-Idu2S(1→4)-α-D-GlcNS6S (II) and ΔIdu2S(1→4)-α-D-GlcNS6S(1→4)-β-D-GlcA(1→4)-α-D-GlcNS6S (III) (Merchant et al., 1985). An additional, pentasulphated, tetrasaccharide was also isolated. This pentasulphated tetrasaccharide IV was co-eluted with tetrasaccharide III on a 10 μm-particle-size s.a.x.h.p.l.c.

Fig. 3. Gradient PAGE of low-Mr, heparin-derived oligosaccharide standards prepared by using g.p.c.–l.p.l.c.

(a) Oligosaccharide samples (40 μl) loaded in every other lane were: 1, decasaccharides (50 μg); 2, octasaccharides (40 μg); 3, hexasaccharides (30 μg) containing V; 4, tetrasaccharides (20 μg) containing II and III and IV; 6, disaccharide (20 μg) containing only 1; and 7, the reaction mixture (200 μg) at 100% completion. Bromophenol Blue (BB) was loaded in lane 4. Only the bottom half of the gel is shown. (b) A scan of lane 7; the peaks corresponding to the low-Mr standards dp 2–10 are indicated.

column (Merchant et al., 1985); however, tetrasaccharide III and IV could be cleanly resolved on a 5 μm-particle-size s.a.x.h.p.l.c. column (Fig. 4). The structure of tetrasaccharide IV was assigned using two-dimensional 1H n.m.r. (Linhardt et al., 1986a) and 13C n.m.r. as ΔIdu2S(1→4)-α-D-GlcNS(1→4)-α-L-Idu2S(1→4)-α-D-GlcNS6S. The hexasaccharide tetrasaccharide II migrated the slowest among the major tetrasaccharides, whereas pentasulphated tetrasaccharides III and IV
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of the oligosaccharides each dp (dp 10-20), are marked with arrows. They were isolated and re-subjected to s.a.x.h.p.l.c. to obtain the high-Mr oligosaccharides shown in Fig. 5. Compounds I–V are labelled.

co-migrated and could not be resolved by gradient-gel electrophoresis (Fig. 3). The major septasulphated hexasaccharide (V), AIdu2S(1-*4)-a-D-GlcNS6S(1-4)-

a-L-Idu(1-4)-a-D-GlcNAc6S(1-4)-

a-D-GlcNS3S6S (Linhardt et al., 1986a), was the most highly charged hexasaccharide and migrated slowest of all the hexasaccharides observed at 100% reaction completion (Fig. 3).

Enzymic depolymerization was interrupted before reaction completion by removal of samples followed by quenching the reaction by heating at 100 °C for 10 min. Samples were examined with and without heating by both s.a.x.h.p.l.c. and gradient PAGE and were found to result in identical product profiles.

Partial enzymic depolymerization of heparin followed by purification of oligosaccharides on s.a.x.h.p.l.c. afforded high-Mr oligosaccharides (Fig. 4). Isolation of the major high-Mr oligosaccharides (Fig. 4) and re-application on s.a.x.h.p.l.c. or g.p.c.-h.p.l.c. resulted in a single symmetrical peak at the expected retention time. Electrophoresis of these oligosaccharides was performed and the results were compared with the banding pattern obtained for a heparin depolymerization carried to 30% reaction completion (Fig. 5). To characterize the high-Mr oligosaccharides, plots of $K_v$ (measured by g.p.c.-h.p.l.c.) and migration distance (measured on gradient PAGE) versus log $M_r$ were constructed (Fig. 6). The $M_r$ values for disaccharide I and tetrasaccharide II were known. The $M_r$ values for oligosaccharides dp 6–20 were estimated by sequentially adding disaccharide I residues on to a tetrasaccharide II backbone. The $K_v$ and migration-distance-versus-log-$M_r$ plots were nearly coincident and were linear between dp 6–20 with correlation coefficients ($r$) of 0.98 and 0.9996 respectively.

Porcine mucosal heparin and bovine kidney heparan sulphate were depolymerized with heparinase and heparitinase respectively, and the products were subjected to gradient PAGE. The parent polymers failed to show banding (lane 1, Figs. 7a and 7b); however, even at 10% reaction completion, considerable banding of high- and low-Mr heparin-derived oligosaccharides was observed (Fig. 7a). Optimum banding patterns (i.e. the greatest number of sharp, well-defined, bands) of higher-Mr heparin-derived oligosaccharides were observed at 30% reaction completion (Fig. 7a). After complete enzymic depolymerization with heparinase, the major bands observed were those of the disaccharide I, tetrasaccharides II, III and IV and the hexasaccharide V. Moreover, the hexasaccharide V appeared to be produced in large quantities only in the last 30% of the heparinase reaction (Fig. 7a). Commercially prepared flavobacterial heparinase and purified heparinase resulted in identical product profiles, as measured by both gradient PAGE and s.a.x.h.p.l.c.

Heparan sulphate showed a different profile, in which intermediate-Mr fragments form rapidly, but do not result in observable low-Mr fragments until very late in the reaction. However, it is possible that the staining may not detect any low-Mr, non-sulphated oligosaccharide. Heparan sulphate contained considerable amounts of high-Mr oligosaccharides, which were undiminished by repeated treatment with heparitinase (Fig. 7b). Partially depolymerized chondroitin 4- and 6-sulphates showed more than 24 sharp bands. The leading band migrated 4 cm further than Bromophenol Blue, which itself
co-migrated with the eighth band at 12.5 cm (results not shown).

**DISCUSSION**

Heparin and heparan sulphate depolymerized by flavobacterial lyases may be resolved into bands of oligosaccharides ranging from dp 2 to greater than dp 20. The resolution of these bands requires the use of both a high-density pore-gradient gel and a stacking gel. Gradient PAGE can be used to determine dp and has the advantages of high resolution, small sample requirements, rapid simultaneous analysis of multiple samples, and detection based upon dye binding without the presence of a u.v. chromophore being required. The purification of higher-$M_r$ oligosaccharides by s.a.x.h.p.l.c. and the correlation of their elution times from g.p.c.-h.p.l.c. with their assigned dp suggests that s.a.x.h.p.l.c. also represents a useful method of preparing and analysing heparin oligosaccharides. This method has the advantages of high resolution, small sample requirements and detection based on the presence of a u.v. chromophore.

Comparison of resolutions achieved by both g.p.c.-l.p.l.c. and g.p.c.-h.p.l.c. to those of gradient PAGE and s.a.x.h.p.l.c. illustrates the superiority of these latter two techniques for analysis. In addition to resolution of oligosaccharides having different dp (i.e. disaccharides, tetrasaccharides, hexasaccharides etc.), it is often necessary to resolve oligosaccharides having the same dp but different sequence (i.e. tetrasaccharides II, III and IV). G.p.c.-l.p.l.c. is clearly inadequate in these regards, giving only a single broad peak for the heparin-derived tetrasaccharides (Fig. 2). The resolution of low-$M_r$ oligosaccharides, of a given dp, by gradient PAGE is best illustrated with the example of the tetrasaccharides. Migration distance is seemingly effected primarily by $M_r$ and not by charge-to-mass ratio. Trisulphated disaccharide I migrated ahead of the pentasulphated and hexasulphated tetrasaccharides III, IV and II. Hexasulphated tetrasaccharide II and disaccharide I have
Electrophoretic fractionation of heparin-derived oligosaccharides

Disaccharide I, tetrasaccharide II, hexasaccharide V and higher oligosaccharides prepared by s.a.x.h.p.l.c. are represented. The two major high-M₆ oligosaccharides (dp 10–20) were eluted from g.p.c.–h.p.l.c. at the same retention time. The octasaccharide and decasaccharide mixtures prepared by g.p.c.–l.p.l.c. (not shown) had \( K_w \) values nearly identical with those of the individual octasaccharide and decasaccharide components prepared by s.a.x.h.p.l.c.

![Figure 6](image)

**Fig. 6.** \( \log M_r \) and dp plotted against \( K_w \) (determined by g.p.c.–h.p.l.c., ○······○) and migration distance (determined by gradient PAGE, □□□□□)

Identical charge-to-mass ratios, yet the molecules are easily resolved. Pentasulphated tetrasaccharides (III and IV) migrate ahead of the hexasulphated tetrasaccharide II and have a lower charge-to-mass ratio. G.p.c.–h.p.l.c. fails to resolve high-M₆ oligosaccharides that have the same dp but a different primary structure. These oligosaccharides are clearly resolved on gradient PAGE (Fig. 5, lanes 1–12), further confirming the superior resolution that is obtained with gradient PAGE. The linear relationship between migration distance and \( \log M_r \) has been reported for both PAGE (Hampson & Gallagher, 1984) and gradient PAGE (Slater, 1969). The plot for the high-M₆ oligosaccharides (dp 6–20) shows good linearity and correlates well with the plot of \( K_w \) against \( \log M_r \) obtained using g.l.c.–h.p.l.c. (Fig. 6). These coincident curves suggest that the major repeating bands observed, on gradient PAGE, each differ by a single disaccharide unit. These observations are in complete agreement with the previously reported results obtained for hyaluronic acid, chondroitin 4- and 6-sulphates and dermatan sulphate (Hampson & Gallagher, 1984; Cowman et al., 1984).

The superior resolution of this PAGE system can be attributed to the use of high-density pore-gradient gels, as conventional isocratic acrylamide gels were unable to resolve both high- and low-M₆ components. The use of a stacking gel was essential to obtain banding of heparin-and heparan sulphate-derived oligosaccharides. Chondroitin sulphate-derived oligosaccharides were also prepared and were similarly resolved into discrete bands on the high-density gradient gel. Very few minor bands were observed, confirming early observations (Hampson & Gallagher, 1984; Cowman et al., 1984) that there is low heterogeneity, or sequence variability, in the chondroitin sulphate polymer.

The purification of high-M₆ oligosaccharides by s.a.x.h.p.l.c. after partial enzymic digestion provides a method for obtaining larger, more homogeneous, heparin oligosaccharides than previously isolated. Analysis of these oligosaccharides on high-density gradient gels with dye staining of bands permitted a purity analysis which was independent of the presence of a u.v. chromophore. Purity analysis of high-M₆ oligosaccharides by s.a.x.h.p.l.c. and high-pore-density gradient gels will be an essential prerequisite to their sequencing and biological-activity testing (Sharath et al., 1985; Beck et al., 1986; Merchant et al., 1986; Linhardt et al., 1986).

Characterization of the gel separation using oligosaccharide standards establishes gradient PAGE as a method of choice for the analysis of unknown heparin-derived oligosaccharides. Caution must be exercised not to simply count the bands of partial or complete enzymic depolymerizations to determine dp. This approach could result in reading errors, either from the non-revealment or non-resolution of a band, the absence of an oligosaccharide of a particular dp or the presence of
multiple bands having the same dp. The use of s.a.x.h.p.l.c. to prepare low- and high-$M_r$ oligosaccharides followed by size determination using g.p.c.–h.p.l.c. currently represents the best method to prepare standards to eliminate these potential ambiguities.

Kinetic profiles of heparin depolymerization by heparinase, and heparan sulphate depolymerization by heparitinase, reveal that the two polymers are degraded to different extents. More high-$M_r$ oligosaccharides are obtained at reaction completion from the heparan sulphate depolymerization than from the heparin depolymerization. Heparin is depolymerized to predominantly one disaccharide (I), three tetrasaccharides (II, III and IV) and one hexasaccharide (V), all of known sequence. The kinetic profile also reveals that hexasaccharide (V) is liberated late in the reaction, perhaps due to the different staining intensities of hexasaccharides or the resistance of heparinase-cleavable linkages containing GlcNS3S6S in place of the usual GlcNS6S residue (Linhardt et al., 1986a). Kinetic analysis of these data should add to a further understanding of the action pattern (Linhardt et al., 1982) and specificity of this important class of enzymes, the polysaccharide lyases (Linhardt et al., 1986b).

In conclusion, analysis of complex, polydisperse, heterogeneous, glycosaminoglycans may be accomplished by their enzymic depolymerization and electrophoresis on a high-density gradient polyacrylamide gel containing a stacking gel. In addition, s.a.x.h.p.l.c. can be used to prepare high-$M_r$ oligosaccharide standards particularly useful for characterizing the PAGE separation. These techniques should prove invaluable in sequencing complex glycosaminoglycans.

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