Biochem. J. (1988) 251, 597–608 (Printed in Great Britain)

Oligosaccharide mapping of heparan sulphate by polyacrylamide-gradient-gel electrophoresis and electrotransfer to nylon membrane

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A new method that we have called 'oligosaccharide mapping' is described for the analysis of radiolabelled heparan sulphate and other glycosaminoglycans. The method involves specific enzymic or chemical scission of polysaccharide chains followed by high-resolution separation of the degradation products by polyacrylamide-gradient-gel electrophoresis. The separated oligosaccharides are immobilized on charged nylon membranes by electrotransfer and detected by fluorography. A complex pattern of discrete bands is observed covering an oligosaccharide size range from degree of polymerization (d.p.) 2 (disaccharide) to approximately d.p. 40. Separation is due principally to differences in M_r, though the method also seems to detect variations in conformation of oligosaccharide isomers. Resolution of oligosaccharides is superior to that obtained with isocratic polyacrylamide-gel-electrophoresis systems or gel chromatography, and reveals structural details that are not accessible by other methods. For example, in this paper we demonstrate a distinctive repeating doublet pattern of iduronate-rich oligosaccharides in heparitinase digests of mouse fibroblast heparan sulphate. This pattern may be a general feature of mammalian heparan sulphates. Oligosaccharide mapping should be a valuable method for the analysis of fine structure and sequence of heparan sulphate and other complex polysaccharides, and for making rapid assessments of the molecular distinctions between heparan sulphates from different sources.

INTRODUCTION

The heparan sulphates are a family of complex polysaccharides that are widely distributed in Nature. They have been identified in all animal tissues, where they are located mainly at cell-surface membranes or in the extracellular matrix and they are thus strategically positioned to mediate interactions between cells and their microenvironment (Kraemer, 1979; Hay, 1981; Gallagher & Hampson, 1984; Höök *et al.*, 1984; Fransson *et al.*, 1986). Heparan sulphates have been implicated in a diverse range of biological functions, including growth control, the regulation of cell adhesion and morphology, lipid metabolism and anticoagulant activities (for review see Gallagher *et al.*, 1986). This functional variety reflects the structural diversity that has been identified in the polysaccharide chains.

Variability in the structure of heparan sulphate arises from extensive post-polymeric modifications of the non-sulphated precursor, heparan, which consists of alternate GlcNAc and GlcA residues (Lindahl *et al.*, 1986; Lindahl & Kjellén, 1987). These modifications include the conversion of some GlcNAc units into $GlcNSO_3$, epimerization of GlcA to IdoA and the formation of ester (*O*-)sulphated derivatives of sugar residues in the vicinity of *N*-sulphated hexosamines. Analysis of the resulting heparan sulphate polymers from various sources has indicated remarkable similarities in the degree of *N*-sulphation, with approximately half of the hexosamine residues existing in the form of $GlcNSO_3$ residues that are arranged in a predominantly segregated manner along the chain (Winterbourne & Mora, 1981; Gallagher & Walker, 1985). In contrast, the pattern and extent of *O*-sulphation appears to display considerable heterogeneity (Gallagher & Walker, 1985), and may be correlated with cell lineage, cell proliferation and neoplastic transformation (Keller *et al.*, 1980; Winterbourne & Mora, 1981; Fransson *et al.*, 1982; David & Van den Berghe, 1983; Fedarko & Conrad, 1986; Gallagher *et al.*, 1986; Pejler *et al.*, 1987). However, these studies did not provide detailed information on the actual sequence of *N*- and *O*-sulphated sugars within heparan sulphate chains. Such studies are essential in order to gain deeper insights into the molecular distinctions between separate polysaccharide species and to establish their structure–function relationships.

Progress in the analysis of the domain structure of heparan sulphate has been limited by the lack of techniques capable of adequately resolving heparan sulphate oligosaccharides. PAGE has proved to be a useful technique for the separation of glycosaminoglycan oligosaccharides of uniform charge/mass ratio derived from hyaluronic acid, chondroitin sulphate and dermatan sulphate (Cowman *et al.*, 1984; Hampson & Gallagher, 1984; Knudson *et al.*, 1984). However, for co-polymers of non-uniform charge density, such as heparan sulphate, resolution of oligosaccharides was not possible by using isocratic PAGE (Hampson & Gallagher, 1984). More

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; d.p., degree of polymerization (e.g. disaccharide has d.p. 2); GlcA, glucuronic acid; IdoA, iduronic acid; IdoA(2S), iduronic acid 2-sulphate; GlcNAc, *N*-acetylglucosamine; $GlcNSO_3$, *N*-sulphated glucosamine; $GlcNSO_3(6S)$, *N*-sulphated glucosamine 6-sulphate.

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recently Linhardt and colleagues made the important observation that structurally diverse heparin oligosaccharides could be separated by gradient PAGE (Rice *et al.*, 1987), indicating that this type of method can provide the resolution required to separate oligosaccharides derived from complex *N*-sulphated glycosaminoglycans.

In the present paper we describe the application of a different gradient PAGE technique (combined with electrotransfer on to nylon membrane and fluorography) for the analysis of radiolabelled heparan sulphate. This yields a complex banding pattern, which represents an 'oligosaccharide map' of the intact molecule, revealing important structural features and providing a means for making detailed comparisons between various polysaccharide species.

EXPERIMENTAL

Materials

D-[1-^{3}H]Glucosamine (sp. radioactivity 5.8 Ci/mmol), $Na_2{}^{35}SO_4$ (sp. radioactivity 25–40 Ci/mg) and [*N*-*sulphonate*-^{35}S]heparin (sp. radioactivity 17 μCi/mg) were obtained from Amersham International. Heparinase (EC 4.2.2.7), heparitinase (EC 4.2.2.8), chondroitin ABC lyase (EC 4.2.2.4) and chondroitin AC lyase (EC 4.2.2.5) were obtained from Seikagaku Fine Biochemicals, Tokyo, Japan. Cell-culture media were supplied by GIBCO Ltd., with the exception of newborn-calf serum, which was obtained from Flow Laboratories. Sephadex G-50 and DEAE-Sephacel were obtained from Pharmacia Ltd., and Bio-Gel P-6 (200–400 mesh) was from Bio-Rad Laboratories Ltd. A 32 cm vertical slab gel unit (SE620) was supplied by Hoefer Scientific Instruments (San Francisco, CA, U.S.A.), and the Trans-blot system for electrophoretic blotting was obtained from Bio-Rad Laboratories Ltd. Biotrace RP nylon membrane was supplied by Gelman Sciences Ltd., and 3MM paper by Whatman Laboratories Sales Ltd. En^{3}Hance spray surface autoradiography enhancer was obtained from NEN Research Products, Du Pont (U.K.) Ltd. Autoradiography cassettes were supplied by Genetic Research Ltd. X-Omat AR X-ray film and development chemicals were supplied by Kodak Ltd. Ready-Solv EP scintillant was obtained from Beckman RIIC Ltd. Tris, bovine serum albumin, *N*-ethylmaleimide, phenylmethanesulphonyl fluoride, *NNN'N'*-tetramethylethylenediamine, 6-aminohexanoic acid, soya-bean trypsin inhibitor, testicular hyaluronidase (EC 3.2.1.35), heparin (from pig intestinal mucosa; ammonium salt) and heparan sulphate (from bovine kidney) were all supplied by Sigma Chemical Co. Ltd. Acrylamide and *NN'*-methylenebisacrylamide (Electran grade) were obtained from BDH Chemicals. All other reagents and chemicals used were of AnalaR or AristaR grade from BDH Chemicals.

Cell cultures

Confluent cultures of foetal human skin fibroblasts were provided by Dr. A. Schor; the cultures were maintained at 37 °C (5% CO_2 in air) in Eagle's Minimal Essential Medium supplemented with 15% (v/v) donor-calf serum, 2 mM-glutamine, 1 mM-sodium pyruvate, non-essential amino acids, penicillin (100 i.u./ml) and streptomycin (100 μg/ml). An embryonic mouse fibroblast cell line (210C parent clone) described previously (Winterbourne & Mora, 1981) was provided by Dr. D. J. Winterbourne, and cultured as described in that reference except that 10% (v/v) donor-calf serum was used instead of foetal-calf serum. Confluent cultures of bovine aortic endothelial cells were provided by Dr. D. West, and were maintained at 37 °C (5% CO_2 in air) in Dulbecco's Minimal Essential Medium supplemented with 10% (v/v) newborn-calf serum, 2 mM-glutamine, penicillin (100 i.u./ml) and streptomycin (100 μg/ml). Extracts of cultured human foetal liver cells were provided by Dr. M. Gordon.

Radiolabelling and extraction of glycosaminoglycans

Confluent cultures were incubated for 24–48 h with $Na_2{}^{35}SO_4$ (20–50 μCi/ml) or [^{3}H]glucosamine (10–20 μCi/ml). The medium was removed and the cell layers were carefully washed twice with warm (37 °C) Dulbecco's phosphate-buffered saline A. The combined medium and rinsings were centrifuged (200 *g* for 10 min) and the supernatant was stored at −20 °C. The cell layers were given a further rinse with Dulbecco's phosphate-buffered saline A (which was discarded) and then extracted by incubation for 16 h at 4 °C in 1% (v/v) Triton X-100 and 6 M-urea in Dulbecco's phosphate-buffered saline A containing a cocktail of proteinase inhibitors (5 mM-EDTA, 5 mM-*N*-ethylmaleimide, 5 mM-benzamidine hydrochloride, 5 mM-6-aminohexanoic acid, 0.25 mM-phenylmethanesulphonyl fluoride, 0.1 mg of soya-bean trypsin inhibitor/ml and 0.1 mg of bovine serum albumin/ml). The resulting extract was centrifuged (200 *g* for 10 min) to remove any unsolubilized material, and the supernatant was stored at −20 °C.

Preparation of intact heparan sulphate chains

Medium and cell-layer extracts were subjected to initial purification by ion-exchange chromatography. Small Poly-prep chromatography columns (Bio-Rad Laboratories Ltd.) packed with 1 ml of DEAE-Sephacel were used. Samples were applied to the column and washed through with 0.15 M-NaCl in 20 mM-sodium phosphate buffer, pH 6.8, followed by 0.25 M-NaCl in the same buffer (to elute contaminating proteins and hyaluronic acid). Remaining glycosaminoglycans (principally heparan sulphate and chondroitin sulphate/dermatan sulphate) were then step-eluted with 1 M-NaCl in 20 mM-sodium phosphate buffer.

The resulting material was dialysed into 25 mM-Tris/acetate buffer, pH 8.0, and digested with chondroitin ABC lyase (0.25 unit/ml) for 60 min at 37 °C. Samples were then heated at 100 °C for 2 min to inactivate the enzyme. Heparan sulphate chains were released from the protein cores by base/borohydride elimination. Samples were adjusted to 50 mM-NaOH/1 M-$NaBH_4$ and incubated for 16 h at 45 °C; they were then neutralized by addition of acetic acid. Chrondroitin ABC lyase digestion products and protein-core fragments were removed by step elution from DEAE-Sephacel as described above. The 1 M-NaCl-eluted fractions were desalted on small Sephadex G-50 columns (bed volume approx. 6 ml). Void-volume fractions, corresponding to intact heparan sulphate chains, were concentrated by centrifugal evaporation and stored at −20 °C.

Dermatan sulphate chains were also isolated from the cell-layer extract of human skin fibroblasts. Samples

were prepared as described for heparan sulphate except that they were treated with low-pH HNO_2 (see below) instead of chondroitin ABC lyase.

Degradation of glycosaminoglycans

Heparitinase (heparan sulphate lyase; heparanase) was used at a concentration of 40 munits/ml in 100 mM-sodium acetate buffer, pH 7.0, containing 0.1 mM-calcium acetate and 1 mg of bovine kidney heparan sulphate/ml as carrier. Samples were incubated at 43 °C for 1 h. Heparinase (heparin lyase) was used at a concentration of 20 munits/ml in the same buffer as for heparitinase but containing 1 mg of heparin/ml as carrier instead of heparan sulphate. Samples were incubated at 30 °C for 2 h. In both cases parallel digests, containing unlabelled heparan sulphate or heparin only, were carried out to monitor the progress of the reaction. Increase in absorbance at 232 nm was measured to ensure that the end point of depolymerization was reached with each batch of digests. HNO_2 deaminative cleavage was carried out by using the low-pH method of Shively & Conrad (1976). Samples were dried down by centrifugal evaporation, reconstituted in 10 μl of 1 M-HNO_2 solution and incubated for 15 min at 20 °C. The reaction was stopped by addition of 10 μl of 1 M-Na_2CO_3, and the samples were diluted to a total volume of 50 μl for electrophoretic analysis. Testicular hyaluronidase digestion of hyaluronic acid was performed in 0.1 M-sodium acetate buffer, pH 5.0, containing 0.15 M-NaCl. Partial digestion was achieved by incubation of a sample (50 μl) with 200 units of enzyme at 37 °C for 10 min; the reaction was stopped by heating at 100 °C for 2 min. Chondroitin AC lyase digestion of metabolically radiolabelled dermatan sulphate was carried out at 37 °C for either 10 or 30 min in 25 mM-sodium acetate buffer, pH 6.0, with 1 unit of enzyme/ml. The reaction was stopped by heating at 100 °C for 2 min.

Gel chromatography

Gel chromatography of glycosaminoglycan oligosaccharide fragments was performed on a Bio-Gel P-6 column (100 cm × 1 cm) equilibrated and eluted with 0.25 M-NH_4HCO_3 at a flow rate of 6 ml/h; 1 ml fractions were collected, and small portions were taken for scintillation counting of radioactivity in Ready-Solv EP scintillant. Fractions corresponding to sized oligosaccharides were pooled, concentrated by repeated centrifugal evaporation and analysed by gradient PAGE.

Preparation of polyacrylamide-gradient gels

Gradient PAGE gels were prepared by using the discontinuous buffer system described originally by Ornstein (1964) and Davis (1964). This consists of a combination of a resolving gel (in 0.375 M-Tris/HCl buffer, pH 8.8), a stacking gel (in 0.125 M-Tris/HCl buffer, pH 6.8) and electrophoresis buffer composed of 25 mM-Tris/192 mM-glycine, pH 8.3.

Polyacrylamide-linear-gradient resolving gels (T 20–30% acrylamide) were prepared by use of a three-channel peristaltic pump as follows. High-concentration gel solution [61.9 ml composed of T 30%/C 5% acrylamide and 10% (v/v) glycerol in resolving gel buffer] and low-concentration gel solution (61.8 ml composed of T 20%/C 0.5% acrylamide in resolving gel buffer) were prepared and degassed. [% T refers to the total concentration (w/v) of acrylamide monomer (i.e. acrylamide plus methylenebisacrylamide); % C refers to the concentration (w/v) of cross-linker (methylenebisacrylamide) relative to total monomer.] Then 10% (w/v) ammonium persulphate was added to these solutions (50 μl and 150 μl respectively), followed by 10 μl of *NNN'N'*-tetramethylethylenediamine. The solutions were mixed thoroughly and then placed in the mixing and limit chambers respectively of the gradient-making apparatus. One channel of the pump was used to transfer low-concentration gel solution from the limit chamber into the mixing chamber (containing high-concentration gel solution and a stirrer bar), and the other two channels were used to simultaneously pump the gradient into two separate Hoefer SE620 gel units (32 cm × 16 cm × 1.5 mm). Since all three channels were run at the same rate (3 ml/min), a linear gradient was formed between the glass plates, from the bottom (maximum gel concentration, T 30%/C 5%) upwards to the top (minimum gel concentration, T 20%/C 0.5%). The unpolymerized gel was then overlaid with resolving-gel buffer solution, and polymerization occurred from the top of the gel downwards.

Gradient PAGE of glycosaminoglycan oligosaccharides

Immediately before electrophoresis the gel surface was rinsed three times with stacking-gel buffer (0.125 M-Tris/HCl buffer, pH 6.8). The stacking-gel solution (T 5%/C 0.5% acrylamide in stacking-gel buffer) was then prepared and degassed, and 200 μl of 10% (w/v) ammonium persulphate and 20 μl of *NNN'N'*-tetramethylethylenediamine were added to initiate polymerization. The solution was applied to the top of the resolving gel, and a 15 × 5 mm-well former comb was inserted between the glass plates. After polymerization (15 min) the comb was removed and the wells were rinsed three times with electrophoresis buffer, after which the gel unit was placed into the electrophoresis tank.

Oligosaccharide samples (5–50 μl) containing approx. 10% (v/v) glycerol and a trace quantity of Phenol Red were carefully layered on to the bottom of the wells with a microsyringe. Marker samples (5–10 μl) containing trace quantities of Bromophenol Blue and Phenol Red in 10% (v/v) glycerol were also run on each gel. Electrophoresis was then performed as follows. Samples were initially run into the gel at 150 V for 30 min, followed by electrophoresis at 300 V for 16 h, and finally 1000 V for 1 h (total approx. 6000 V·h). Under these conditions the Phenol Red marker dye migrates to within approx. 3 cm of the bottom of the resolving gel. Throughout the run heat was dissipated by using a heat exchanger with circulating tap water (10–15 °C).

Electrotransfer to nylon membrane

Immediately after termination of electrophoresis the gel was removed from between the glass plates and cut into two pieces (19 cm from the bottom of the resolving gel; this was necessary because of the limited size of the transfer apparatus), and the stacking gel was removed, before equilibration in transfer buffer (25 mM-Tris/192 mM-glycine, pH 8.3) for 15 min to allow any gel swelling to occur. The gel was then placed on to a sheet of filter paper (Whatman 3MM) soaked in transfer buffer, and a sheet of nylon membrane (Biotrace RP) presoaked in transfer buffer was laid on top of the gel. A

further sheet of presoaked nylon membrane was placed on top of the first layer, followed by a sheet of soaked filter paper; in each case care was taken to avoid entrapment of air bubbles. Good contact between the gel and nylon membrane was ensured by gently rolling a glass tube over the top piece of filter paper. The whole assembly was then sandwiched between two Scotch-Brite pads in a cassette, which was inserted into the transfer tank containing transfer buffer at 4 °C. Transfer was carried out at 25 V (constant voltage, current approx. 100 mA) for 5 h at 4 °C with nylon membrane on the anodic side of the gel. This resulted in complete transfer of the dye markers from the gel to the nylon membrane. In some cases different transfer conditions were used, and these are described in the Figure legends where appropriate.

Fluorography

Radiolabelled glycosaminoglycans transferred on to nylon membrane were detected by fluorography. The membranes were vacuum-dried at 75 °C for 2 h, and then sprayed evenly with En^{3}Hance surface autoradiography enhancer and allowed to dry. They were then placed in an autoradiography cassette with a sheet (20 cm × 25 cm) of Kodak X-Omat AR X-ray film (pre-exposed to 0.15 A_{540} unit). Exposure was carried out for a suitable period (typically 5–10 days) at −70 °C, after which the film was unpacked while still at −70 °C and developed with LX-24 developer, indicator stop bath and FX-40 liquid fixer (all from Kodak Ltd.). Note that only the lower 19 cm of the gel is shown in each fluorograph (corresponding approximately to the 23–30 % region of the gradient). Developed films were scanned with a Shimadzu scanning densitometer at 550 nm with the use of a 0.05 mm × 2 mm slit width.

RESULTS

Gradient PAGE and electrotransfer of glycosaminoglycan oligosaccharides

The inability of PAGE systems utilizing isocratic polyacrylamide gels to separate heparan sulphate oligosaccharides (Hampson & Gallagher, 1984) led us to investigate the application of gradient PAGE methodology to this problem. Oligosaccharides of heparan sulphate ranging in size from disaccharide (d.p. 2) to dodecasaccharides (d.p. 12) were prepared by limit heparitinase digestion of metabolically labelled polysaccharide and chromatography on Bio-Gel P-6 (Fig. 1*a*). Heparitinase, which is believed to cleave mainly the hexosaminidic linkages between GlcNAc/GlcNSO$_3$ and GlcA (Linker & Hovingh, 1977; Silverberg *et al.*, 1985; Nader *et al.*, 1987; see the Discussion section), produces a mixture of oligosaccharides that is dependent on the distribution of susceptible linkages within the intact molecule. Individual oligosaccharide peaks were pooled, concentrated and then analysed by gradient PAGE and electrotransfer on to nylon membrane, along with an unfractionated sample of enzyme-digested material (Fig. 2*a*). Fluorography revealed multiple species in all gel-filtration fractions, with each fraction containing two major bands and two to four minor components. The major bands seemed to be a series of repeating doublets. Thus preparations that give single peaks on gel filtration display significant heterogeneity when subjected to the more discerning M_r separations that can be achieved with gradient PAGE. All the bands detected in the individual gel-filtration peaks were readily observed by direct separation of the unfractionated heparitinase digest (compare track 7 with tracks 1–6 in Fig. 2*a*). Therefore this method of gel electrophoresis can be used as a means of high-resolution analysis of very complex mixtures of oligosaccharides. These 'oligosaccharide maps' provide a fingerprint of the molecular structure of the heparan sulphate chains. Quantitative information can readily be obtained by scanning densitometry of the developed X-ray film (Fig. 2*b*).

The gradient PAGE system also showed high resolving

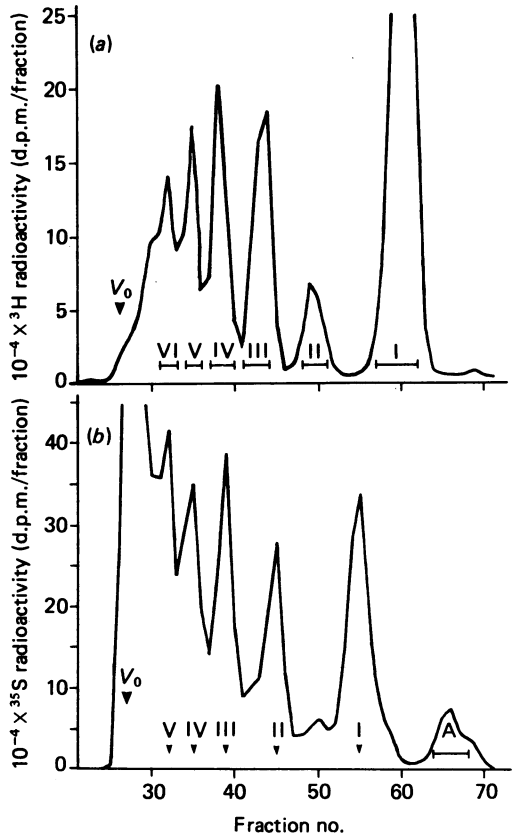

Fig. 1. Fractionation of heparan sulphate and heparin oligosaccharides by gel filtration on Bio-Gel P6

(*a*) ^{3}H-labelled heparan sulphate isolated from 210C mouse fibroblast medium was degraded with heparitinase, and the resulting oligosaccharide mixture was fractionated on a Bio-Gel P6 gel-filtration column (100 cm × 1 cm) eluted with 0.25 M-NH_4HCO_3 at a flow rate of 6 ml/h (V_0, void volume); 1 ml fractions were collected. Fractions were pooled as indicated by the bars for analysis by gradient PAGE (see Fig. 2), and correspond to: I, disaccharides (d.p. 2); II, tetrasaccharides (d.p. 4); III, hexasaccharides (d.p. 6); IV, octasaccharides (d.p. 8); V, decasaccharides (d.p. 10); VI, dodecasaccharides (d.p. 12). (*b*) ^{35}S-labelled heparin was degraded with heparinase, and the resulting oligosaccharide mixture was fractionated on a Bio-Gel P6 column as in (*a*). Single fractions were taken as indicated by the arrows for analysis by gradient PAGE (see Fig. 3), and correspond to: I, disaccharides (d.p. 2); II, tetrasaccharides (d.p. 4); III, hexasaccharides (d.p. 6); IV, octasaccharides (d.p. 8); V, decasaccharides (d.p. 10); A, free [^{35}S]sulphate.

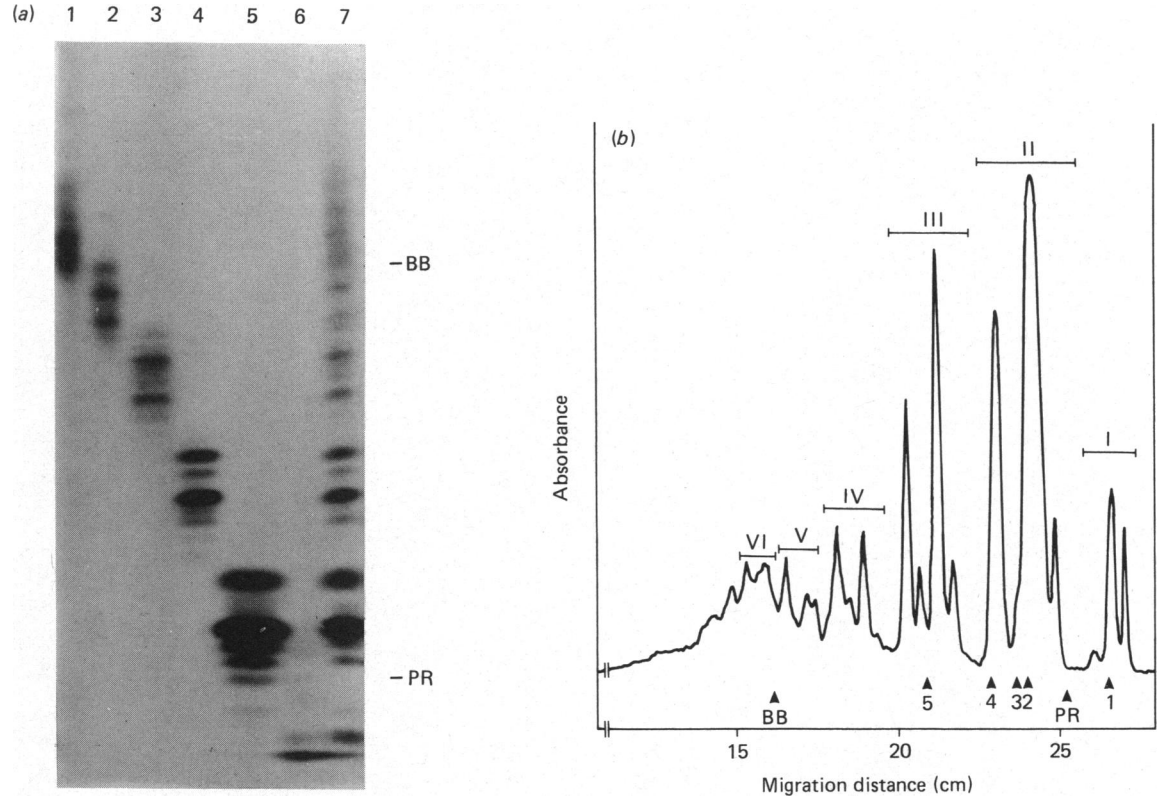

Fig. 2. Fractionation of heparan sulphate oligosaccharides by gradient PAGE and electrotransfer to nylon membrane

(*a*) Heparan sulphate oligosaccharides fractionated on Bio-Gel P6 (Fig. 1*a*) were concentrated, separated by gradient PAGE and transferred to nylon membrane for fluorography. Oligosaccharide peaks (100000 d.p.m. of ^3H radioactivity) loaded in each track were: 1, dodecasaccharides (d.p. 12); 2, decasaccharides (d.p. 10); 3, octasaccharides (d.p. 8); 4, hexasaccharides (d.p. 6); 5, tetrasaccharides (d.p. 4); 6, disaccharides (d.p. 2). Track 7 was loaded with the complete reaction mixture (300000 d.p.m. of ^3H radioactivity). Only the lower 19 cm of the gel is shown (corresponding approximately to the 23–30% portion of the gradient). The migration positions of Bromophenol Blue (BB) and Phenol Red (PR) were as indicated. (*b*) Densitometric scan of track 7, with each group of bands corresponding to the gel-filtration peaks as indicated. The migration distances of five structurally characterized heparin oligosaccharides (Rice *et al.*, 1987) are also shown: 1, trisulphated disaccharide; 2, pentasulphated tetrasaccharide A; 3, pentasulphated tetrasaccharide B; 4, hexasulphated tetrasaccharide; 5, heptasulphated hexasaccharide.

power for the analysis of heparin oligosaccharides prepared by Bio-Gel P-6 chromatography (Fig. 1*b*) of limit heparinase digests. Heparinase selectively cleaves hexosaminidic linkages between $GlcNSO_3(6S)$ and IdoA(2S) residues (Linker & Hovingh, 1977; Merchant *et al.*, 1985). These oligosaccharide preparations (d.p. 2–10) each contained a major band and several minor components (Fig. 3).

The principal basis for the separation of the complex saccharides seemed to be M_r. There was an inverse correlation between the molecular size of the oligosaccharide peaks obtained by gel filtration and the electrophoretic mobility of each group of bands resolved from these peaks. The components in the disaccharide fraction (d.p. 2) were the most mobile, migrating just ahead of the Phenol Red dye marker, whereas the largest fractions (d.p. 12) yielded the least-mobile saccharides, which migrated just behind the Bromophenol Blue marker dye. The multiple bands identified in each oligosaccharide fraction from the gel-filtration column presumably represent components that vary in their degree of sulphation. We were also provided with structurally characterized heparin fragments (d.p. 2–6) by Dr. R. J. Linhardt, and these migrated to positions predicted from our own PAGE data (Fig. 2*b*). It may be noted that two pentasulphated tetrasaccharides (A and B) could be separated. Their structures were $\Delta IdoA(2S)\alpha1\rightarrow4GlcNSO_3(6S)\alpha1\rightarrow4GlcA\beta1\rightarrow4GlcNSO_3(6S)$ and $\Delta IdoA(2S)\alpha1\rightarrow4GlcNSO_3\alpha1\rightarrow4IdoA(2S)\alpha1\rightarrow4GlcNSO_3(6S)$ respectively (see Rice *et al.*, 1987). This result indicates that different patterns of sulphation and/or uronate composition can also affect the migration properties of oligosaccharides in this system.

We confirmed that M_r was the principal determinant of separation by preparing a wide range of dermatan sulphate oligosaccharides that resolved into a ladder-like series of major bands (d.p. 4–40; Figs. 4*a* and 4*b*), and gave a linear plot for $\log M_r$ against migration distance (Fig. 4*c*). Slight deviations from linearity occurred only with the low-M_r oligosaccharides, probably because they had not yet reached their pore limits (see the Discussion section). Anomalous migration of low-M_r oligosaccharides that occurs in isocratic gels (Hampson & Gallagher, 1984) was not apparent on the gradient gels. The series of major and minor dermatan sulphate oligosaccharides revealed by using this system indicated

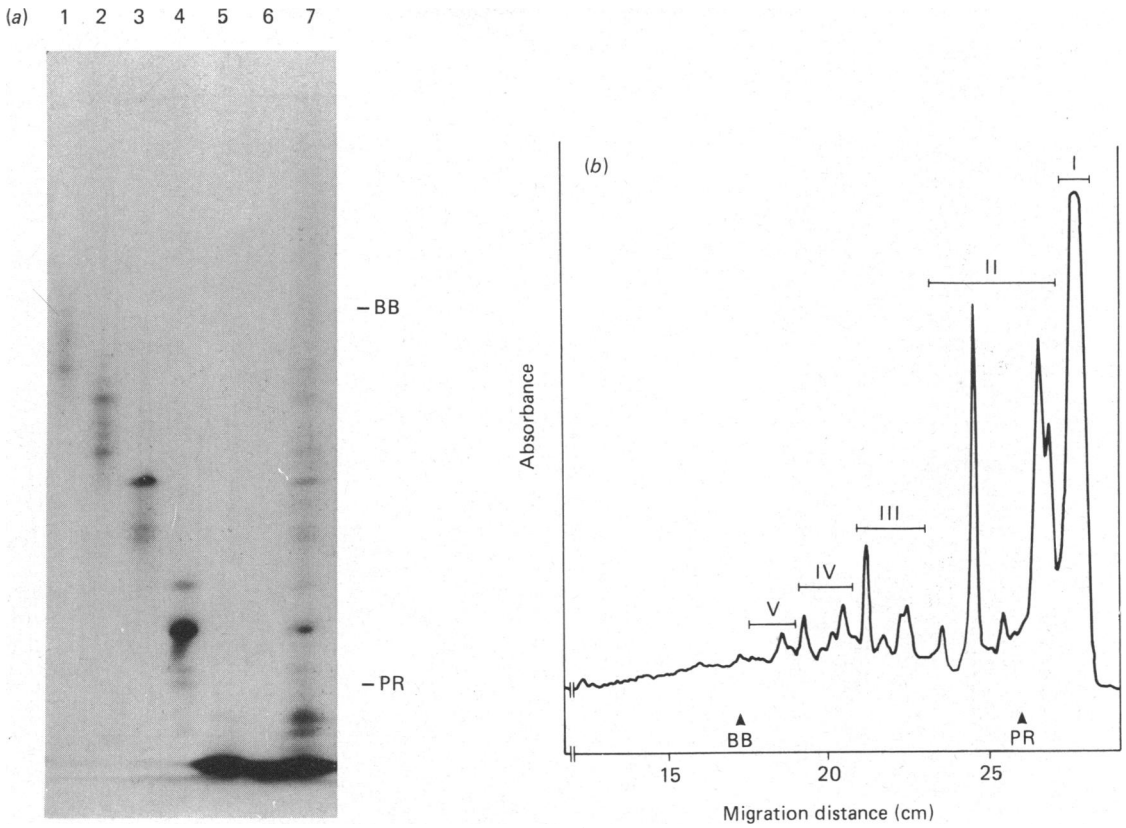

Fig. 3. Fractionation of heparin oligosaccharides by gradient PAGE and electrotransfer to nylon membrane

(*a*) Heparin oligosaccharides fractionated on Bio-Gel P6 (Fig. 1*b*) were concentrated, separated by gradient PAGE and transferred to nylon membrane for fluorography. Oligosaccharide peaks (20000 d.p.m. of ^{35}S radioactivity) loaded in each track were: 1, decasaccharides (d.p. 10); 2, octasaccharides (d.p. 8); 3, hexasaccharides (d.p. 6); 4, tetrasaccharides (d.p. 4); 5, disaccharides (d.p. 2); 6, free [^{35}S]sulphate. Track 7 was loaded with the complete reaction mixture (50000 d.p.m. of ^{35}S radioactivity). The migration positions of Bromophenol Blue (BB) and Phenol Red (PR) were as indicated. (*b*) Densitometric scan of track 7, with each group of bands corresponding to the gel-filtration peaks as indicated.

a degree of resolution not achieved with previous PAGE methods.

Oligosaccharide mapping of heparitinase-treated heparan sulphates

The value of oligosaccharide mapping for carrying out comparative analyses of heparan sulphate is illustrated in Fig. 5, in which metabolically ^{35}S-labelled polysaccharides from different sources have been digested with heparitinase and fractionated by gradient PAGE. A complex series of bands was observed in each sample, and some basic similarities between them were clearly evident (Fig. 5). However, each heparan sulphate exhibited a unique overall pattern, since differences in the quantities of specific bands present could be identified. For example, an oligosaccharide in the d.p. 4–6 range (labelled A) was prominent in the patterns of liver (tracks 3 and 4) and endothelial (track 5) heparan sulphates, but was only present as a minor component in mouse fibroblast material (tracks 1 and 2). Another example is the series of bands (labelled B) in the d.p. 2–4 range that are more prominent components in endothelial medium heparan sulphate (track 5) than in all the other samples. Differences in the maximum size of fragments generated by heparitinase were also evident. Within a particular cell type (i.e. mouse fibroblast or foetal liver) the patterns generated from medium and cell-layer extracts were broadly similar, but not identical, since differences in the quantities of specific bands present could be observed (compare tracks 1 and 2 and tracks 3 and 4 respectively). In addition, the maximum size of fragment generated by heparitinase digestion is apparently greater in medium samples (tracks 1 and 3) compared with the corresponding cell-layer or cell-surface samples (tracks 2 and 4 respectively) from the same cell type. These results indicate that the described methodology can reveal unique structural differences between heparan sulphates that are dependent on both the cell type and the cellular compartment from which the molecules are derived.

Oligosaccharide mapping of HNO_2-treated heparan sulphate

Deaminative scission of heparan sulphate with low-pH HNO_2 produces an interesting series of oligosaccharides for analysis. This reagent hydrolyses the glycosidic linkages only of $GlcNSO_3$ units, releasing *N*-sulphate groups as inorganic sulphate. Ester (*O*-)sulphate groups are unaffected in this reaction. It follows that HNO_2 treatment of ^{35}S-labelled heparan sulphate will produce a series of oligosaccharides in which only the *O*-sulphated species will be radiolabelled. By contrast, radiolabel will be present in all of the oligosaccharides

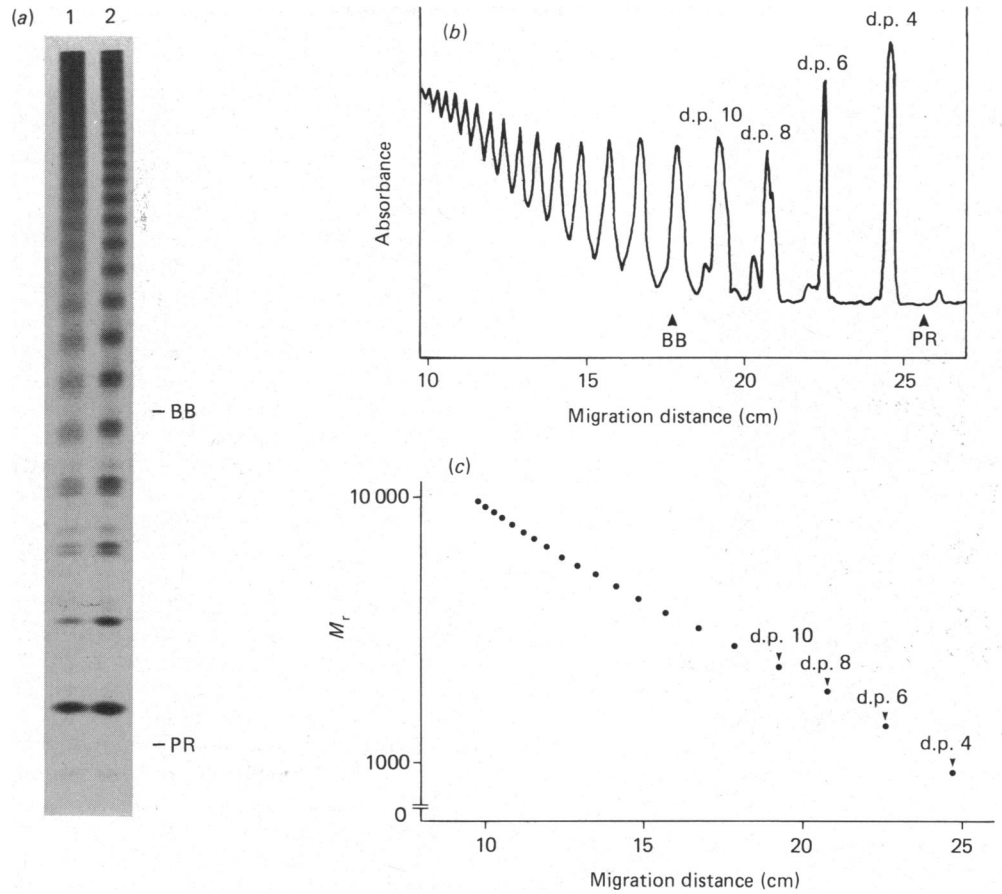

Fig. 4. Fractionation of dermatan sulphate oligosaccharides by gradient PAGE and electrotransfer to nylon membrane

(*a*) ^{35}S-labelled dermatan sulphate isolated from the cell layer of human skin fibroblasts was partially degraded with chondroitin AC lyase, and the resulting oligosaccharides were separated by gradient PAGE and transferred to nylon membrane for fluorography (track 1, 10 min partial degradation; track 2, 30 min degradation). Note that in order to improve the resolution of larger oligosaccharides the total volt-hours applied were increased to 6800, resulting in migration of disaccharides off the gel. The migration positions of Bromophenol Blue (BB) and Phenol Red (PR) were as indicated. (*b*) Densitometric scan of track 2. Positions of the major peaks d.p. 4–10 (tetrasaccharides to decasaccharides) are indicated. (*c*) A plot of $\log M_r$ against migration distance for the series of major oligosaccharides (d.p. 4–40) shown in (*b*), each differing in size by a single disaccharide unit. Each unit is assumed to have an M_r of 460 (i.e. the M_r of a monosulphated disaccharide).

generated from HNO_2 scission of heparan sulphate labelled with [^{3}H]glucosamine. In Fig. 6(*a*) tracks 1 and 2 show the patterns for HNO_2-treated ^{35}S-labelled and ^{3}H-labelled heparan sulphate respectively. The latter yields a complex series of bands of size range d.p. 2 to approx. d.p. 24 (Fig. 6*c*), the larger fragments (d.p. 6 and above) being produced from regions where two or more *N*-acetylated disaccharides occur in sequence. *O*-Sulphated sugars are almost entirely restricted to di- and tetra-saccharides (Fig. 6*b*). As these fragments are derived from regions of high *N*-sulphate content (Gallagher & Walker, 1985), the result confirms data from a number of other studies (reviewed in Gallagher *et al.*, 1986) that *N*- and *O*-sulphate groups are in close proximity in heparan sulphate.

Experimental variables

Initial experiments were carried out with a continuous buffer system (10 mM-Tris/5 mM-acetate/0.5 mM-EDTA, pH 7.8) and a resolving gel containing a gradient of both acrylamide and cross-linker (methylenebisacrylamide). Since the concentration of cross-linker relative to total monomer (designated % C) affects pore size (Hames, 1981; Rodbard *et al.*, 1972), superimposition of a gradient of cross-linker (0.5% to 5% C) upon an increasing total acrylamide concentration gradient (T 20%–30%) results in a gel with a very steep pore-size gradient. This system proved capable of improved resolution of heparan sulphate and heparin oligosaccharides compared with isocratic gels. However, we subsequently found that a combination of the discontinuous buffer system originally described by Ornstein (1964) and Davis (1964) with the above gradient resolving gel provided superior resolution of glycosaminoglycan oligosaccharides. Heparan sulphate fragments varying in size from d.p. 2 (disaccharide) to approx. d.p. 20 (Fig. 2) were clearly resolved under these conditions. Larger heparan sulphate oligosaccharides were only partially resolved, but it is probable that such components (and in principle even larger saccharide chains extending up to the length of intact glycosaminoglycans) should be separable by use of polyacrylamide gradients that extend over a lower concentration range. Indeed, by using different combinations of total volt-hours applied and

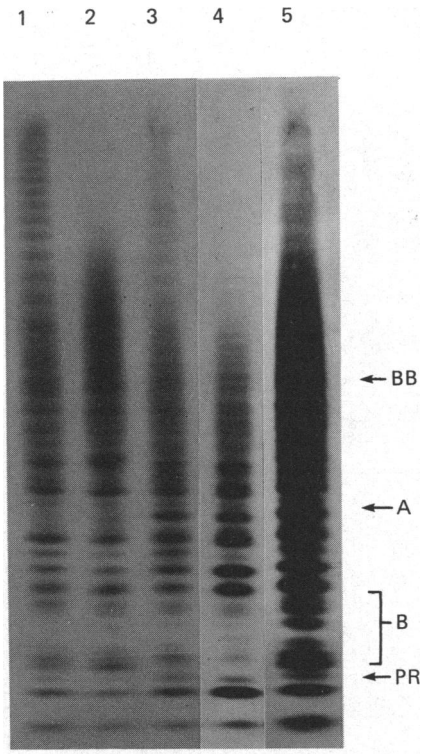

Fig. 5. Oligosaccharide mapping of heparan sulphates from various sources after degradation with heparitinase

^{35}S-labelled heparan sulphates isolated from various sources were subjected to limit degradation with heparitinase, and the resulting oligosaccharides were separated by gradient PAGE, transferred to nylon membrane and detected by fluorography. The migration positions of Bromophenol Blue (BB) and Phenol Red (PR) were as indicated. For the significance of the oligosaccharide bands labelled A and B see the text. Oligosaccharides (approx. 25000 d.p.m./track) loaded in each track originated from: 1, 210C mouse fibroblast medium; 2, 210C mouse fibroblast cell layer (Triton extract); 3, foetal liver medium; 4, foetal liver trypsin extract; 5, bovine aortic endothelial-cell medium.

gradient-gel compositions, it is possible to devise systems for optimal separation of various size ranges of oligosaccharides. We should also emphasize that unlabelled glycosaminoglycan fragments separated by this gradient PAGE technique can be detected by using staining methods described by others (Cowman *et al.*, 1984; Min & Cowman, 1986; Rice *et al.*, 1987).

Electrotransfer of radiolabelled glycosaminoglycan oligosaccharides from gradient gels on to nylon membrane was investigated as a potentially useful method for their immobilization and detection. This technique was found to be more sensitive than fluorography of the gel itself (results not shown). A time course for the transfer of hyaluronic acid and heparin oligosaccharides (i.e. unsulphated and highly sulphated oligosaccharides) from a gradient gel to a double layer of nylon membrane was established (Fig. 7). The results highlighted a number of factors that affect transfer. The M_r of oligosaccharides affects the rate of transfer, with small fragments transferring rapidly (in less than 1 h) compared with the 3–6 h required for more complete transfer of high-M_r fragments. Under the voltage conditions used it appears that unsulphated fragments (hyaluronic acid oligosaccharides) do not readily bind to the nylon membrane, since a gradual loss from both the first (Fig. 7*a*) and second (Fig. 7*b*) layers of nylon was observed. This effect is also clearly M_r-dependent, with lower-M_r fragments being lost most rapidly. However, of the sulphated fragments (heparin oligosaccharides) only the disaccharide band begins to transfer (though incompletely) through to the second layer of nylon (Fig. 7*b*). Similar results were obtained for heparan sulphate oligosaccharides; only disaccharides (and some tetrasaccharides) began to transfer partially through to the second layer after a 5 h transfer at 25 V (results not shown). The conditions described in the Experimental section were chosen as a suitable standard transfer protocol, and two layers of nylon were used routinely to minimize the risk of loss of fragments.

When using the described methodology for mapping analysis with polysaccharide lyases, careful attention must be paid to the digestion conditions in order to ensure that the end point of depolymerization is reached. By stopping the action of heparitinase at different times it was possible to assess the progress of degradation of heparan sulphate in molecular terms (Fig. 8*a*). Densitometric scans at 20% (Fig. 8*b*) and 95% (Fig. 8*c*) of reaction completion provide kinetic profiles that indicate a complex pattern of enzyme action. It is clear that anomalous mapping patterns could result from incomplete depolymerization, preventing accurate comparison of different samples. Addition of unlabelled substrate to each sample and simultaneous digestion of different samples under identical conditions should minimize these potential differences. These results also show that oligosaccharide mapping may be applied to the study of the action patterns of these enzymes.

DISCUSSION

The results shown demonstrate that the gradient PAGE technique provides high resolution of heparan sulphate and heparin oligosaccharides. The separation of heparin fragments achieved is similar to that described by Rice *et al.* (1987) using a different gradient PAGE method, and is superior to both gel filtration and previous PAGE systems using isocratic polyacrylamide gels (Cowman *et al.*, 1984; Hampson & Gallagher, 1984). The high resolving power of this technique is due to the combination of a discontinuous buffer system with a pore-size-gradient polyacrylamide resolving gel. The technique is also applicable to glycosaminoglycan oligosaccharides of relatively uniform charge/mass ratio such as dermatan sulphate, providing a higher degree of resolution than isocratic PAGE methods (see Fig. 4). Separation appears to be dependent predominantly on M_r (Figs. 2 and 4), with oligosaccharides exhibiting a near-linear relationship between electrophoretic mobility and $\log M_r$, in agreement with observations made by others (Cowman *et al.*, 1984; Hampson & Gallagher, 1984; Rice *et al.*, 1987). The multiplicity of bands observed for heparan sulphate and heparin oligosaccharides probably represent species differing in their degree of sulphation, and thus being separated by virtue of differences in M_r. However, analysis of well-characterized heparin oligosaccharides indicated that gradient PAGE will separate oligosaccharide isomers as a conse-

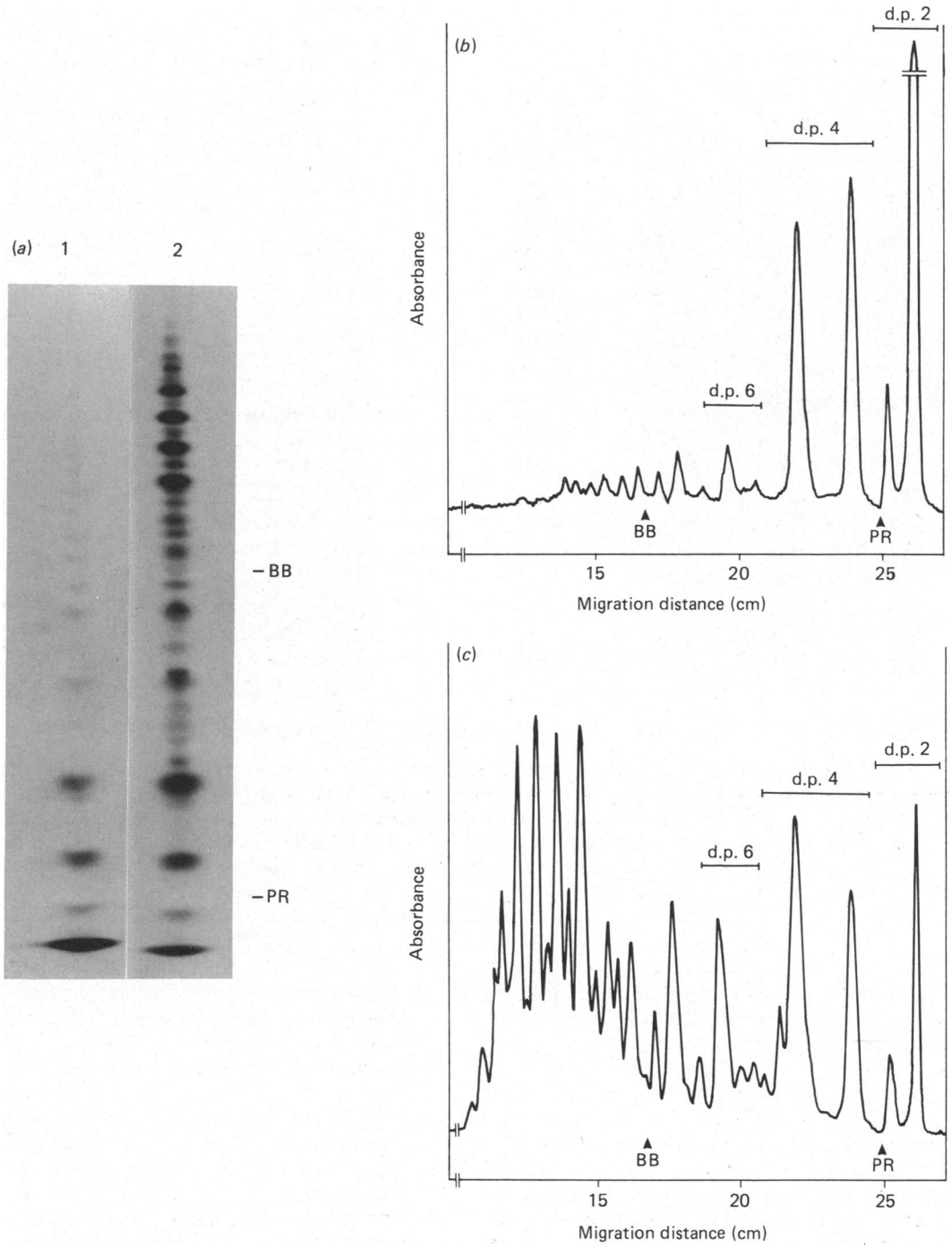

Fig. 6. Oligosaccharide mapping of heparan sulphate by degradation with low-pH HNO_2

(*a*) Heparan sulphate isolated from mouse fibroblast medium was subjected to complete low-pH HNO_2 hydrolysis, and the resulting oligosaccharides were separated by gradient PAGE, transferred to nylon membrane and detected by fluorography. Tracks 1 and 2 were ^{35}S-labelled (25000 d.p.m.) and ^{3}H-labelled (250000 d.p.m.) heparan sulphate respectively. The migration positions of Bromophenol Blue (BB) and Phenol Red (PR) were as indicated. (*b*) and (*c*) Densitometric scans of tracks 1 and 2 respectively.

quence (we assume) of differences in conformation between the individual species (Fig. 2).

Although we have restricted the majority of the results presented here to a fixed gradient PAGE and electrotransfer system, the flexibility of these techniques should be noted. In particular, the pore-size-gradient resolving gel and voltage conditions can be varied to provide optimum resolution of different size ranges of oligosaccharides. It is likely that the best resolution of fragments will be obtained in systems using gels with pore-size gradients devised to allow extended total volt-hour voltage conditions that bring all the oligosaccharides in the size range of interest to their pore limits. At this point molecules experience greatly

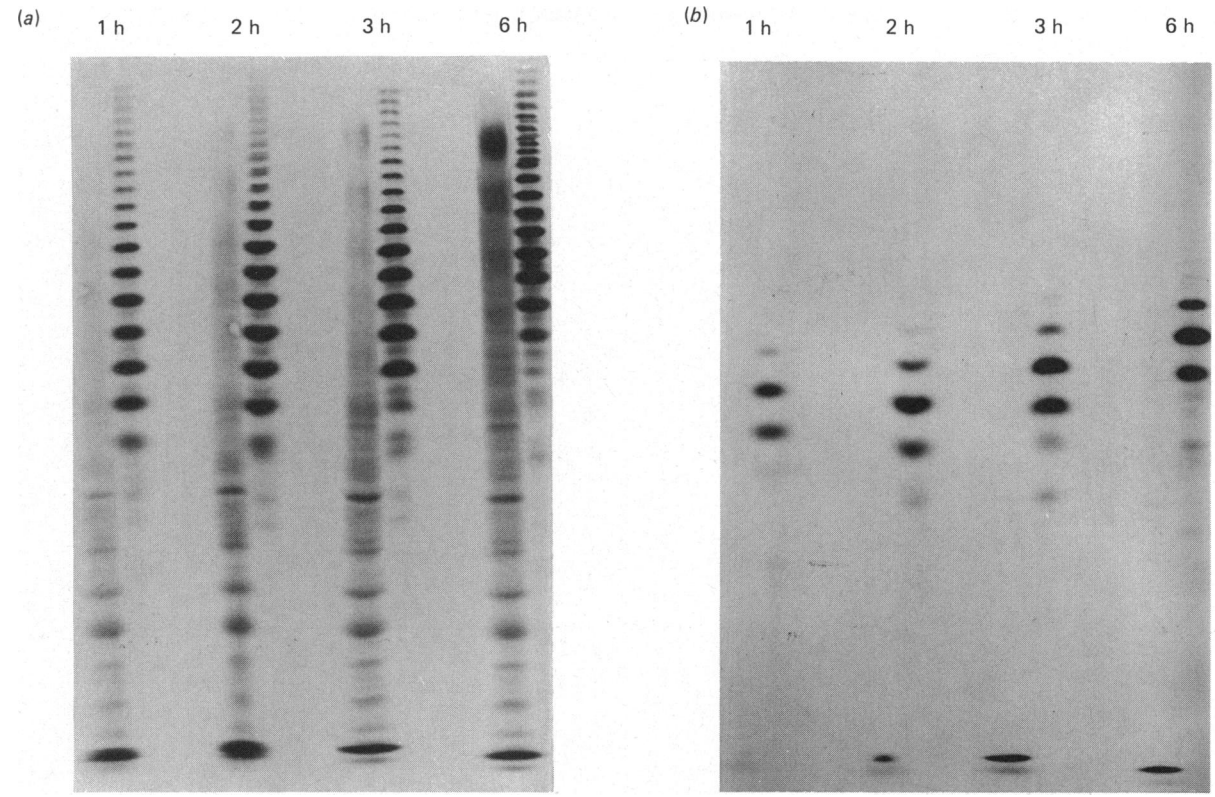

Fig. 7. Time course of electrotransfer of glycosaminoglycan oligosaccharides to nylon membrane

Oligosaccharides from ^{3}H-labelled hyaluronic acid (partially digested with testicular hyaluronidase) and ^{35}S-labelled heparin (heparinase-digested) were separated by gradient PAGE in four separate pairs of adjacent gel tracks. After completion of electrophoresis the gel was cut into strips corresponding to these paired lanes, and each was subjected to electrotransfer to a double layer of nylon membrane [(*a*) first layer; (*b*) second layer]. After different transfer times (1 h, 2 h, 3 h and 6 h) each set of nylon membrane sheets was removed and dried for fluorography. Each panel of two lanes represents the fluorograph of heparin (left) and hyaluronic acid (right) oligosaccharides.

diminished electrophoretic mobility and compress into well-resolved bands (Margolis & Kenrick, 1968). However, it is possible that extended electrophoresis may diminish resolution of some oligosaccharides that are separated under the 'dynamic' conditions prevailing before their pore limit is reached. Detection of resolved radiolabelled oligosaccharides can be achieved by using standard gel-fluorography methods, but the electrotransfer technique that we have developed provides a more rapid, convenient and sensitive method for their detection. The transfer of resolved carbohydrate molecules to an immobilizing matrix is noteworthy, as it represents a novel application of blotting methodology.

A number of applications of these techniques can be envisaged. Oligosaccharide mapping studies of heparan sulphate and heparin are clearly possible, providing a rapid and reproducible method for the simultaneous comparison of multiple samples (see below). More detailed structural studies of these polymers will be possible when structures are assigned to all the distinct species resolved by gradient PAGE. The possibility of conducting binding studies on immobilized oligosaccharides should lead to a better understanding of their structure–function relationships. Gradient PAGE will also provide accurate M_r values for both oligosaccharides and intact polysaccharides, and it may be very useful for the study of polymer-level modification reactions in the synthesis of heparan sulphate, dermatan sulphate and heparin. In addition, these methods can be applied to the study of the action patterns and specificity of the polysaccharide-degrading enzymes (see Rice *et al.*, 1987).

Oligosaccharide mapping revealed unique structural differences between heparan sulphates that were dependent on both cell type and the cellular compartment from which they were derived (Fig. 5). These oligosaccharide maps varied most conspicuously in the 'minor bands', suggesting that specific areas of the polysaccharide are targets for the generation of fine-structural variability.

As well as providing a simple and rapid method for comparing heparan sulphates on the basis of gel banding patterns, the oligosaccharide maps can be used to elucidate important features of the molecular structure and organization of the polysaccharide chains. This is well illustrated in Fig. 2, in which a repeating doublet pattern is observed in the main oligosaccharide size classes following heparitinase treatment of ^{3}H-labelled heparan sulphate from mouse fibroblasts. Though the substrate specificities of commercial heparitinase are not firmly established, it is likely that the preparation we used will cleave heparan sulphate between hexosamines and glucuronic acid, i.e. GlcNAc/GlcNSO$_3$$\alpha$1→4GlcA, irrespective of 6-*O*-sulphation of the amino sugar (Linker & Hovingh, 1977; Nader *et al.*, 1987). [Heparitinase

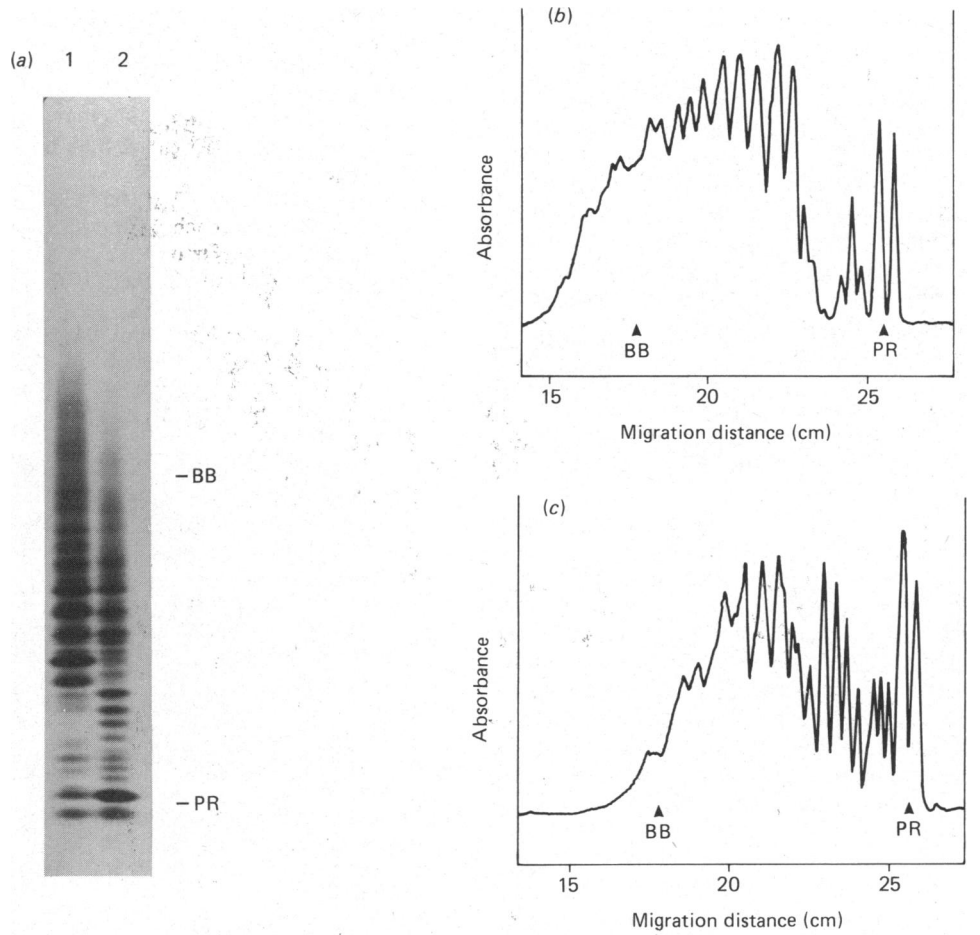

Fig. 8. Kinetic profile of heparitinase degradation of heparan sulphate

(*a*) ^{35}S-labelled heparan sulphate isolated from human skin-fibroblast medium was partially digested with heparitinase, and the resulting oligosaccharides were separated by gradient PAGE and transferred to nylon membrane for fluorography. Partial digestions (track 1, 30 s; track 2, 30 min) represent approx. 20% and 95% of reaction completion respectively. Note that in order to improve the resolution of larger oligosaccharides the total volt-hours applied were increased to 6800, resulting in migration of disaccharides off the gel. The migration positions of Bromophenol Blue (BB) and Phenol Red (PR) were as indicated. (*b*) and (*c*) Densitometric scans of tracks 1 and 2 respectively.

digests of heparan sulphate consistently yield at least two sulphated disaccharides (see, e.g., Figs. 2 and 5). According to published data cited in the text, one of these is likely to be $\Delta GlcA\beta1\rightarrow4GlcNSO_3$. To account for additional sulphated disaccharides, the simplest assumption is that the hexosamine units can be 6-*O*-sulphated.] The hexosaminidic linkage between $GlcNSO_3$ and IdoA ($GlcNSO_3\alpha1\rightarrow4IdoA$) is resistant to the enzyme. At a 43 °C incubation temperature the heparitinase used in this study has very little activity against heparin (5% of the activity of heparinase). Therefore the general formula for heparitinase-derived fragments is:

$$\Delta GlcA\beta1\rightarrow4[GlcNSO_3\alpha1\rightarrow4IdoA]_n\alpha1\rightarrow4GlcNAc/GlcNSO_3$$

The sizes of the major heparitinase-derived ^{3}H-labelled oligosaccharides (Figs. 1*a* and 2) ranged from disaccharides, d.p. 2 (i.e. $n = 0$), to d.p. 12 ($n = 5$), indicating that the maximum length of the major IdoA-containing disaccharide repeats in these structures (i.e. n) is equal to 5. However, a small quantity of larger oligosaccharides ($n > 5$) was also noted (Figs. 1 and 2), particularly with ^{35}S-labelled samples (Fig. 5, track 1). The latter may be due to a biasing effect of the radiolabel on fluorography, since these fragments will have a high sulphate group content. Because all the oligosaccharides from d.p. 4 to d.p. 12 contained a doublet of major bands, it is likely that this is due to the rather broad specificity of the enzyme so that either GlcNAc or $GlcNSO_3$ may be found at the reducing ends of the fragments. This remains to be confirmed. Alternatively, the doublets could be due to the presence or the absence of 6-*O*-sulphate groups on hexosamine residues within these oligosaccharides. The minor bands in each oligosaccharide fraction are probably due to other 'isomeric' and/or quantitative variations in sulphation.

Oligosaccharide mapping of heparan sulphate with HNO_2 (Fig. 6) supports the view that *N*- and *O*-sulphate groups are clustered in heparan sulphate (reviewed in Gallagher *et al.*, 1986), in accord with a recently proposed biosynthetic scheme (Lindahl *et al.*, 1986; Lindahl & Kjellén, 1987). As *O*-sulphate groups are key substituents in defining differences in heparan sulphate in relation to cell lineage (Gallagher & Walker, 1985), cell malignancy

and viral transformation (Winterbourne & Mora, 1981; Pejler *et al.*, 1987), antithrombin-binding activity (Marcum & Rosenberg, 1985; Pejler *et al.*, 1987) and nuclear localization (Fedarko & Conrad, 1986), oligosaccharide mapping with the use of HNO_2 scission of ^{35}S-labelled heparan sulphate should be of great value in rapidly identifying small but potentially significant differences in *O*-sulphation patterns of closely related polysaccharides. In addition, oligosaccharide maps generated with heparinase, which cleaves specifically at highly sulphated disaccharide units, i.e. $GlcNSO_3(6S)\alpha1\rightarrow4IdoA(2S)$ (Linker & Hovingh, 1977; Merchant *et al.*, 1985), should reveal further interesting features of the polysaccharide chain, and the sequential use of enzymic and chemical scission methods will be of considerable value in sequencing, for example, the repeating oligosaccharide doublets identified in Fig. 2 (see also Nader *et al.*, 1987).

In summary, oligosaccharides generated by specific enzymic or chemical cleavage of heparan sulphate (and heparin) can be resolved by gradient PAGE and immobilized on nylon membrane. The detected banding patterns represent oligosaccharide maps of the intact polymers, revealing important structural features and providing a means of making detailed comparisons between different polysaccharide species. Furthermore, these techniques will undoubtedly be applicable to the problem of sequencing these complex molecules and therefore elucidation of their structure–function relationships.

We thank Dr. A. Schor, Dr. D. West and Dr. D. J. Winterbourne for provision of cell cultures, Dr. M. Gordon for provision of liver-cell-culture extracts, Mr. J. Deakin for technical assistance, Dr. M. Lyon for helpful advice and for reading the manuscript and Dr. R. J. Linhardt and Dr. K. Rice for the gift of heparin oligosaccharides. We also thank Mrs. P. Jones for secretarial help.

REFERENCES

Cowman, M. K., Slahetka, M. F., Hittner, D. M., Kim, J., Forino, M. & Gadelrab, G. (1984) Biochem. J. **221**, 707–716

David, G. & Van den Berghe, H. (1983) J. Biol. Chem. **258**, 7338–7344

Davis, B. J. (1964) Ann. N.Y. Acad. Sci. **121**, 404–427

Fedarko, N. S. & Conrad, H. E. (1986) J. Cell Biol. **102**, 587–599

Fransson, L.-Å., Havsmark, B. & Chiarugi, V. P. (1982) Biochem. J. **201**, 233–240

Fransson, L.-Å., Carlstedt, I., Cöster, L. & Malmström, A. (1986) Ciba Found. Symp. **124**, 125–142

Gallagher, J. T. & Hampson, I. N. (1984) Biochem. Soc. Trans. **12**, 541–543

Gallagher, J. T. & Walker, A. (1985) Biochem. J. **230**, 665–674

Gallagher, J. T., Lyon, M. & Steward, W. P. (1986) Biochem. J. **236**, 313–325

Hames, B. D. (1981) in Gel Electrophoresis of Proteins: A Practical Approach (Hames, B. D. & Rickwood, D., eds.), p. 4, IRL Press, Oxford

Hampson, I. N. & Gallagher, J. T. (1984) Biochem. J. **221**, 697–705

Hay, E. D. (1981) J. Cell Biol. **91**, 205s–223s

Höök, M., Kjellén, L., Johansson, S. & Robinson, J. (1984) Annu. Rev. Biochem. **53**, 847–869

Keller, K. L., Keller, J. M. & Moy, J. N. (1980) Biochemistry **19**, 2529–2536

Knudson, W., Gundlach, M. W., Schmid, T. M. & Conrad, H. E. (1984) Biochemistry **23**, 368–375

Kraemer, P. M. (1979) in Surfaces of Normal and Malignant Cells (Hynes, R. O., ed.), pp. 149–198, John Wiley and Sons, New York

Lindahl, U. & Kjellén, L. (1987) in The Biology of the Extracellular Matrix: Biology of Proteoglycans (Wight, T. N. & Mecham, R. P., eds.), pp. 59–104, Academic Press, New York

Lindahl, U., Feingold, D. S. & Rodén, L. (1986) Trends Biochem. Sci. **11**, 221–225

Linker, A. & Hovingh, P. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. **36**, 43–46

Marcum, J. A. & Rosenberg, R. D. (1985) Biochem. Biophys. Res. Commun. **126**, 365–372

Margolis, J. & Kenrick, K. G. (1968) Anal. Biochem. **25**, 347–362

Merchant, Z. M., Kim, Y. S., Rice, K. G. & Linhardt, R. J. (1985) Biochem. J. **229**, 369–377

Min, H. & Cowman, M. K. (1986) Anal. Biochem. **155**, 275–285

Nader, H. B., Dietrich, C. P., Buonassisi, V. & Colburn, P. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3565–3569

Ornstein, L. (1964) Ann. N.Y. Acad. Sci. **121**, 321–349

Pejler, G., Bäckström, G., Lindahl, U., Paulsson, M., Dziadek, M., Fujiwara, S. & Timpl, R. (1987) J. Biol. Chem. **262**, 5036–5043

Rice, K. G., Rottink, M. K. & Linhardt, R. J. (1987) Biochem. J. **244**, 515–522

Rodbard, D., Levitov, C. & Chrambach, A. (1972) Sep. Sci. **7**, 705–723

Shively, J. E. & Conrad, H. E. (1976) Biochemistry **15**, 3932–3942

Silverberg, I., Havsmark, B. & Fransson, L.-Å. (1985) Carbohydr. Res. **137**, 227–238

Winterbourne, D. J. & Mora, P. T. (1981) J. Biol. Chem. **256**, 4310–4320

Received 4 September 1987/28 October 1987; accepted 16 December 1987