

## Separation of radiolabelled glycosaminoglycan oligosaccharides by polyacrylamide-gel electrophoresis

Ian N. HAMPSON\* and John T. GALLAGHER†‡

\*Clinical Research and †Cancer Research Campaign Department of Medical Oncology, University of Manchester, Christie Hospital, Wilmslow Road, Manchester M20 9BX, U.K.

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Glycosaminoglycan oligosaccharides generated by treatment of biosynthetically radiolabelled dermatan sulphate and hyaluronic acid with chondroitin AC lyase or testicular hyaluronidase may be resolved into a series of discrete bands by polyacrylamide-gel electrophoresis. Bands were identified by fixation in glacial acetic acid containing 20% (w/v) 2,5-diphenyloxazole followed by fluorography. The bands represented glycans which differed in size by one disaccharide unit. For the larger oligosaccharides (decasaccharides and above) of similar charge: mass ratio, there was a linear relationship between electrophoretic mobility and  $\log M_r$ . However, the smaller species showed anomalous migration patterns. Consideration of the structures of the fragments produced by the different enzyme treatments suggests that copolymeric and homopolymeric oligosaccharides may be separated by polyacrylamide-gel electrophoresis. There are many potential applications of this technique, foremost amongst them being studies on the molecular size heterogeneity and patterns of enzyme-mediated depolymerization of native glycosaminoglycan chains and investigations into rates of polymer chain elongation and post-polymerization modification reactions so essential to glycosaminoglycan function.

Typically, glycosaminoglycans are negatively charged, linear polysaccharides which contain sulphate residues and are covalently linked to protein in their native, proteoglycan form (Lindahl & Höök, 1978). Notable exceptions are hyaluronic acid, which is not sulphated and not linked to protein (Prehm, 1983), and certain forms of keratan sulphate which may be branched in the protein-linkage region. The basic structural unit is a disaccharide which usually consists of an amino-sugar and a hexuronic acid; the keratan sulphate disaccharide contains galactose, not hexuronic acid. The polymer chain is therefore built up of disaccharide repeat sequences, but structural diversity may be conferred upon the macromolecule by variations in both degree and position of sulphate groups and in the configuration of the hexuronate residues which, in glycosaminoglycans such as dermatan sulphate and heparan sulphate,

may be in the *D-gluco* or the *L-ido* configuration. Structural variability arises through an orderly series of post-polymerization modification reactions which result in the conversion of identical non-sulphated disaccharide sequences into more complex sulphated chemical domains. Polymer conversion frequently occurs in a non-uniform manner yielding the so-called copolymeric glycosaminoglycans, which include in the same polysaccharide chain structural features characteristic of the primary biosynthetic product and of the enzymically transformed derivatives (Rodén, 1980; Riesenfeld *et al.*, 1982).

A full understanding of the mechanisms and extent of the modification reactions and the associated biological specificities acquired by the variable regions requires analytical techniques that will resolve glycosaminoglycan oligosaccharides which vary in size by a single disaccharide unit, ideally over an  $M_r$  range from the disaccharide itself to the size of the native chain. Additionally, specific methods of chemical or enzymic cleavage are required to generate fragments derived by

Abbreviation used: IdoA, iduronic acid.

‡ To whom correspondence and reprint requests should be addressed.

attack at defined structural regions of the chain. In this study we describe the application of polyacrylamide-gel electrophoresis to the analysis of glycosaminoglycan oligosaccharides produced by enzymic degradation of copolymeric dermatan sulphate and hyaluronic acid.

Preliminary accounts of this work, in abstract form, have been presented (Hampson & Gallagher, 1983; Gallagher & Hampson, 1984).

## Experimental

### Materials

Human skin fibroblast cultures were kindly supplied by Dr. S. Schor, Department of Medical Oncology, Christie Hospital, Manchester. Sephacryl S-300 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Chondroitin AC lyase (EC 4.2.2.5) was obtained from Seikagaku Fine Biochemicals, Tokyo, Japan. Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) was from Miles Laboratories, Slough, Berks., U.K. Electran grade 1 acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were purchased from BDH, and 2,5-diphenyloxazole was from Koch-Light. X-Omat-S film, 40 cm × 30 cm, was obtained from Kodak and 40 cm × 30 cm autoradiography cassettes were from Genetic Research Instrumentation, Bishops Stortford, Herts., U.K.

### Vertical plate polyacrylamide-gel electrophoresis

The method used was based on that described by Sanger & Coulson (1975). Two alternative electrophoresis buffers were prepared as follows: either a 10-fold concentration of Tris/borate/EDTA (0.9 M-Tris base/0.9 M-boric acid/24 mM-EDTA, pH 8.3) or a 2-fold concentration of Tris/glycine (0.2 M-glycine/2.5 mM-EDTA/5 mM- $\text{NaN}_3$  adjusted to pH 8.9 with solid Tris). These buffers were also prepared in deionized (passed through Amberlite MB-3) 8 M-urea.

### Preparation of polyacrylamide gels

A solution (80 ml) of 15% (w/v) acrylamide/0.065% methylenebisacrylamide was made up in the appropriate dilution of electrophoresis buffer. Polymerization was achieved by addition of 3.5 ml of 2% (w/v) ammonium persulphate and 75  $\mu$ l of tetramethylethylenediamine to the pre-cooled (4°C) acrylamide solution. The plate (dimensions 40 cm × 20 cm × 1 mm) was poured at room temperature and polymerization occurred within 10 min. The plate was allowed to stand for a further 30 min before the slot former (18 slots, 5 mm wide) was removed. Following this, the sample wells were exhaustively rinsed with buffer to

remove unpolymerized acrylamide and the plate was pre-electrophoresed at 12.5 V/cm for a minimum of 5 h.

Samples (maximum volume 10  $\mu$ l) containing 2–3% (w/v) Ficoll and trace quantities of Bromophenol Blue and Phenol Red were carefully layered onto the bottom of prefilled sample wells. Electrophoresis was then performed at 12.5–25 V/cm, depending on the nature of the samples. Electrophoresis times varied from 1.5 to 3.5 h (full details are given in the Figure legends).

### Gel processing for fluorography

The two glass plates were carefully separated, leaving the gel attached to one of these. The gel and plate were immersed in 20% (w/v) 2,5-diphenyloxazole in glacial acetic acid (as used by Pulleyblank & Booth, 1981) at 35°C. The gel was carefully 'teased' off the glass plate and gently agitated. Initially, the 2,5-diphenyloxazole/acetic acid solution caused the gel to become opaque, but over a period of 10 min the gel became transparent. The gel was then transferred back onto the plate and placed in a distilled water bath for 8 min. This rehydration step precipitated the 2,5-diphenyloxazole in the gel matrix. The gel was then layered onto a sheet of Whatman 3MM paper for drying on a conventional vacuum gel dryer.

The completely dry gel was placed in an autoradiography cassette with a sheet (30 cm × 40 cm) of Kodak X-Omat-S which had been pre-exposed to an  $A_{530}$  of 0.1–0.2. Exposure times were typically 6 days at –70°C, after which the film was unpacked while still at –70°C and developed with Kodak D19 developer.

### Enzyme digestions

Chondroitin AC lyase was used at a concentration of 0.3 units/ml in 0.1 M-sodium acetate buffer, pH 6.0 (Hiyama & Okada, 1975). Samples were incubated at 37°C for 4 h. Testicular hyaluronidase was used at a concentration of 40 turbidity-reducing units/ml in 0.1 M-sodium acetate, pH 6.0, containing 0.15 M-NaCl. Samples were usually incubated for 18 h at 37°C, although on some occasions short incubation periods were used to generate glycosaminoglycan fragments with a wide range of  $M_r$  values (see, e.g., Figs. 2 and 6). On such occasions the enzyme was inactivated by heating at 100°C for 5 min. Products of partial enzyme digestions were fractionated by exclusion gel chromatography on Sephacryl S-300 in 4.0 M-guanidinium chloride/50 mM-sodium phosphate buffer, pH 6.5.

### Preparation of radiolabelled glycosaminoglycans and derived oligosaccharides

Biosynthetically radiolabelled hyaluronic acid and copolymeric dermatan sulphate were prepared

from cell cultures of human skin fibroblasts (Gallagher *et al.*, 1983a). Low- $M_r$  oligosaccharides derived by chondroitin AC lyase treatment of these glycosaminoglycans were prepared by exclusion gel chromatography on Bio-Gel P-series columns as described previously (Gallagher *et al.*, 1983a,b).

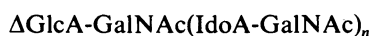
## Results

### *Polyacrylamide-gel electrophoresis of dermatan sulphate oligosaccharides*

In the initial experiments two preparations of radiolabelled copolymeric dermatan sulphate were used. One was a collagen-binding species that was isolated by collagenase treatment of human skin fibroblasts grown on a collagen gel, and the other was a mixed population of collagen-binding and collagen non-binding dermatan sulphates released into the culture medium by identical cell cultures grown on a plastic substratum (Gallagher *et al.*, 1983a). Each preparation was limit digested with chondroitin AC lyase and testicular hyaluronidase by using the enzymes individually, or in alternating sequence (see Fig. 1 legend). Aliquots (10  $\mu$ l) of the digests were applied to polyacrylamide gels. One set of samples was electrophoresed for the full running time (3.5 h) and a second set was loaded 2 h into the run. The longer running times give more effective resolution of high- $M_r$  fragments (with run-off of the smaller oligosaccharides), whereas shorter times allow resolution of low- $M_r$  constituents. Marker dyes showed the relative positions of the oligosaccharides with the different times of electrophoresis. Fluorographic analysis of the gels revealed a ladder-like banding pattern for each sample (Fig. 1). About 70 bands are visible in the Figure, but an additional slower-moving 10–15 faint bands can be seen when viewed by indirect light.

Chondroitin AC lyase cleaves copolymeric dermatan sulphate at GalNAc residues linked ( $\beta$ 1-4) to GlcA, but disaccharides of structure GalNAc-( $\beta$ 1-4)IdoA are resistant to the enzyme, (Malmström *et al.*, 1975). Testicular hyaluronidase also attacks the dermatan sulphate chain at GalNAc-( $\beta$ 1-4)GlcA residues, but this enzyme requires two or more GlcA repeat disaccharide sequences and the major products of enzyme degradation of totally sensitive homopolymeric glycosaminoglycans, such as hyaluronic acid and chondroitin 4/6 sulphates, are the tetra- and hexa-saccharides (Weissmann, 1955; Highsmith *et al.*, 1975).

The general formula for each band generated by chondroitin AC lyase is:



and that for fragments produced by testicular hyaluronidase is:



where  $n$  is the number of disaccharide repeats resistant to the enzymes. In the case of testicular hyaluronidase, each GlcA-GalNAc sequence in the resistant fragment would be flanked by one or more IdoA-GalNAc repeats. It follows that some of the oligosaccharides produced by testicular hyaluronidase may represent copolymeric sequences. These sequences, and the predominantly homopolymeric sequences produced by chondroitin AC lyase, are clearly resolved by the polyacrylamide gels (Fig. 1). In view of the mode of action of these enzymes, and taking note of the fact that DNA and protein of similar conformation and charge density are separated on polyacrylamide gels according to their  $M_r$  values, it seemed reasonable to assume that  $M_r$  was the principal determinant of the mobility of dermatan sulphate (and hyaluronic acid; see later) oligosaccharides. Although this assumption proved to be valid for larger structures (decasaccharides above), smaller species behaved anomalously on polyacrylamide gels.

### *Correlation of oligosaccharide fractionation by polyacrylamide-gel electrophoresis and by exclusion gel chromatography*

When a sample of collagen-binding dermatan sulphate was degraded to disaccharides by treatment with chondroitin ABC lyase, the material gave a relatively broad band on polyacrylamide gels with a mobility that corresponded to the second most mobile species produced by chondroitin AC lyase digestion of the same glycosaminoglycan (arrow in Fig. 1). This indicated that the most mobile species on the gel was not a disaccharide.

To investigate this problem further di-, tetra- and hexa-saccharide fragments produced by chondroitin AC lyase treatment of dermatan sulphate were prepared by chromatography on Bio-Gel P-4 (Gallagher *et al.*, 1983a) and were electrophoresed alongside the unfractionated sample of enzyme-degraded material. The results showed that the most mobile component was the tetrasaccharide and that the mobility of the disaccharide was similar to that of the hexasaccharide (Fig. 2). When the tetrasaccharide was treated with chondroitin ABC lyase to degrade it to disaccharides, its mobility decreased accordingly (Fig. 2). Dermatan sulphate oligosaccharides of size deca-saccharide and above gave electrophoretic mobilities that were directly proportional to the log of their  $M_r$  values (Fig. 3). Deviations from linearity occurred only with the low- $M_r$  constituents, an

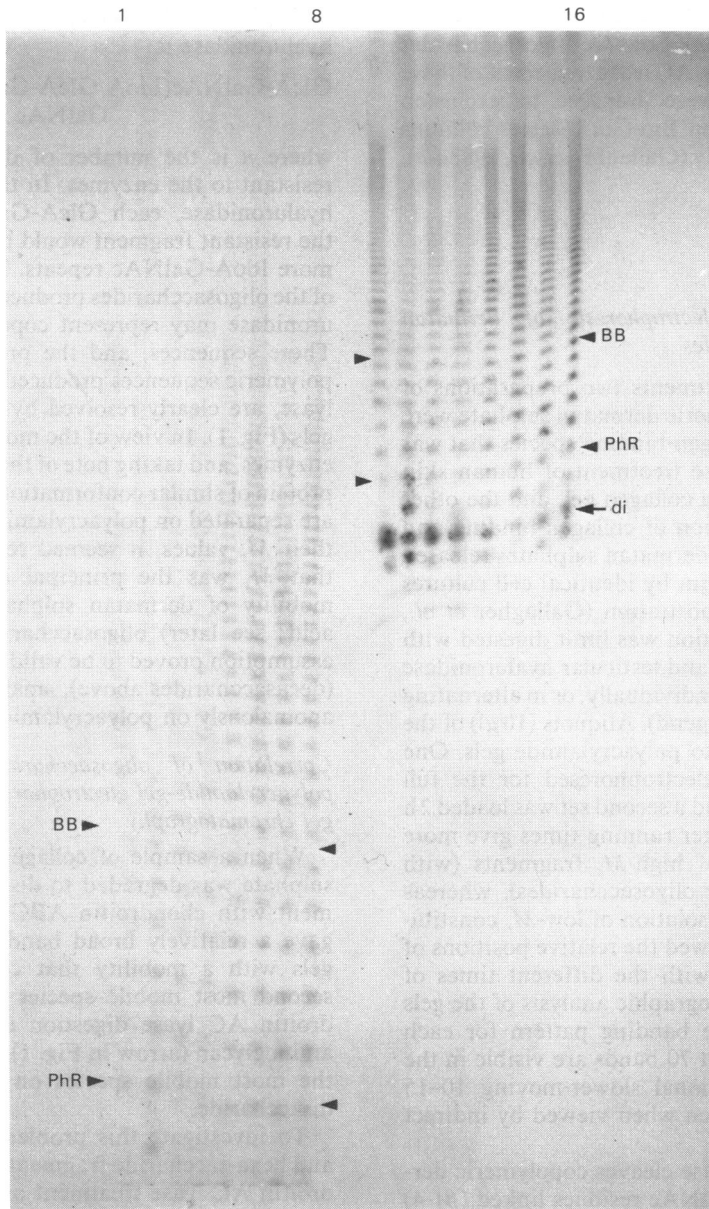


Fig. 1. Polyacrylamide-gel electrophoresis of radiolabelled dermatan sulphate oligosaccharides

Two preparations of dermatan sulphate were used: one was a mixed population of collagen-binding and collagen non-binding material (tracks 1-4 and 9-12 numbered from left to right), the other was a preparation only of collagen-binding dermatan sulphate (tracks 5-8 and 13-16). Each preparation was subjected to prolonged incubation with chondroitin AC lyase, testicular hyaluronidase or combined treatments with the two enzymes (see the Experimental section). Tracks 1-8 were subjected to long running times (3.5 h) to resolve high  $M_r$  fragments; tracks 9-16 were run for 1.5 h to allow detection of low- $M_r$  substituents that are eluted from the gel with the longer electrophoretic periods. Enzyme treatments were as follows: track 1, chondroitin AC lyase; track 2, testicular hyaluronidase; track 3, chondroitin AC lyase followed by testicular hyaluronidase; track 4, testicular hyaluronidase followed by chondroitin AC lyase. Tracks in sequences 5-8, 9-12 and 13-16 were from material given identical enzyme treatments to those described for tracks 1-4. The gels were run in Tris/glycine/EDTA, pH 8.9, at 650 V and 25 mA. Banding patterns in each track can be cross-matched by using the marker dyes Phenol Red (PhR) and Bromophenol Blue (BB). Approx.  $8 \times 10^4$  c.p.m. per track was applied in  $10 \mu\text{l}$ .

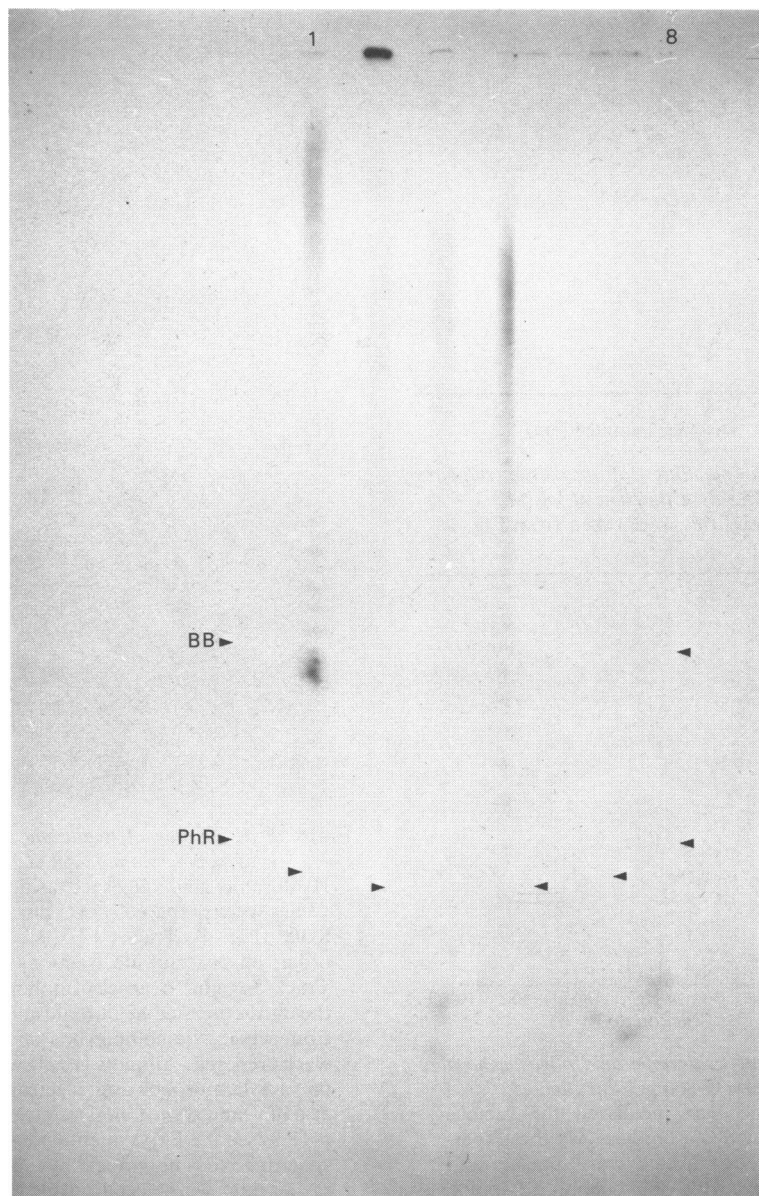


Fig. 2. Polyacrylamide-gel electrophoresis of low- $M_r$  dermatan sulphate oligosaccharides. Oligosaccharides of  $^3\text{H}$ -labelled dermatan sulphate were prepared by fractionation on Bio-Gel P-4 of material treated with chondroitin AC lyase. Tracks are numbered in the sequence 1 (far left) to 8 (far right). Samples were loaded as follows: track 8, disaccharide; track 7, tetrasaccharide; track 6, disaccharides formed by treatment of the tetrasaccharide with chondroitin ABC lyase; track 5, hexasaccharide; track 4, oligosaccharides in the excluded volume ( $V_0$ ) of the Bio-Gel P-4 column. Due to uneven heating effects during the run, samples at the edge (track 8, the disaccharides) run more slowly than samples towards the centre. Although the disaccharide in track 8 appears to be slower moving than that in track 6, reference to the positions of the marker dyes (arrows) illustrates that their relative mobilities are almost identical. Disaccharides (track 8) and hexasaccharides (track 5) have overlapping mobilities. For comparison, unfractionated dermatan sulphate oligosaccharides are shown in track 3 and hyaluronic acid oligosaccharides in track 1. Track 2 shows the smearing effect observed with radiolabelled heparan sulphate oligosaccharides produced by partial nitrous acid (pH 1.5) treatment. Running conditions were as described in Fig. 1. Tracks 1–4 contained  $3.5 \times 10^4$  c.p.m. of  $^3\text{H}$  and tracks 7 and 8 received  $2 \times 10^3$  c.p.m. of  $^3\text{H}$ . Because of the very low levels of radioactivity available, all of the samples were loaded onto tracks 5 and 6 without taking samples for counting.

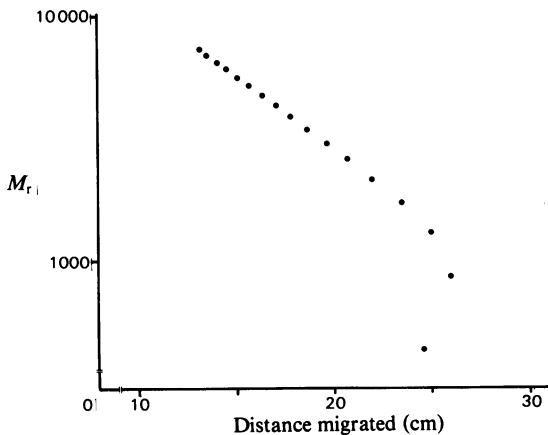


Fig. 3. Electrophoretic mobilities of dermatan sulphate oligosaccharides as a function of  $\log M_r$ . Electrophoretic mobilities were taken from Fig. 1.

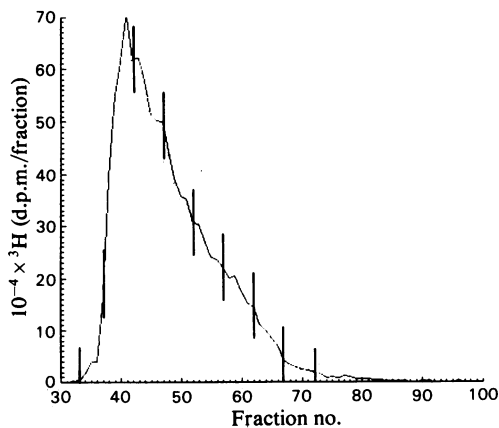


Fig. 4. Fractionation of hyaluronic acid oligosaccharides by Sephacryl S-300 gel filtration

$[^3\text{H}]$ Hyaluronic acid was incubated for various times with testicular hyaluronidase (see the Experimental section). The digests were pooled and applied to a Sephacryl S-300 column (2.5 cm  $\times$  90 cm) eluted with 4.0 M-guanidinium chloride in 50 mM-sodium phosphate buffer, pH 6.5. The flow rate was 10 ml/h. Fractions (4.8 ml) were collected and 100  $\mu$ l of each fraction was taken for radioactivity measurement. As the elution profile shows, a heterogeneous population of hyaluronic acid oligosaccharides was produced. Fractions were pooled as indicated by vertical bars to give preparations of different  $M_r$  values for analysis by polyacrylamide-gel electrophoresis (Fig. 5).

observation confirmed in the accompanying paper (Cowman *et al.*, 1984).

Additional confirmation that the method separates glycosaminoglycan oligosaccharides princi-

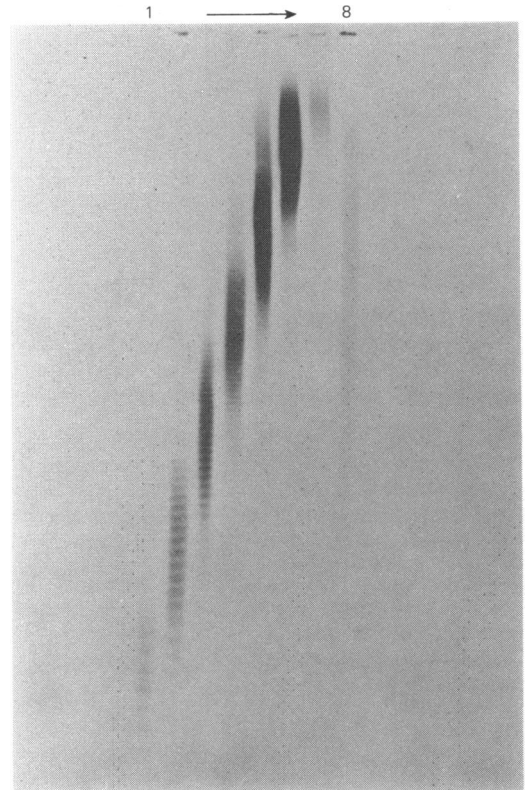


Fig. 5. Fractionation of hyaluronic acid oligosaccharides by polyacrylamide-gel electrophoresis

Hyaluronic acid oligosaccharides of different  $M_r$  values were prepared by gel filtration on Sephacryl S-300 (Fig. 4). Tracks 1-7 (left to right) show, in order, oligosaccharide fractions of increasing  $M_r$ . Track 8 (right) is an electrophoretic separation of the unfractionated hyaluronic acid oligosaccharides from which the components shown in tracks 1-7 were prepared. Aliquots (10  $\mu$ l) were applied to the polyacrylamide gel and electrophoresed for 3.5 h at 650 V and 35 mA in Tris/glycine/EDTA buffer, pH 8.9 (see the Experimental section). Bands corresponding to individual hyaluronic acid oligosaccharides were visualized by fluorography.

pally on the basis of molecular size was demonstrated by using products of partial testicular hyaluronidase treatment of  $[^3\text{H}]$ hyaluronic acid. Glycan populations of different molecular size range were prepared from the digests by chromatography on Sephacryl S-300 (Fig. 4) and electrophoresed on polyacrylamide gels (Fig. 5). The range of electrophoretic mobilities exhibited by each sample correlated directly with the relative molecular size range of the component species, the largest fragments having the lowest mobilities and the smallest fragments the highest mobilities. This

result also demonstrates that polyacrylamide-gel electrophoresis will separate non-sulphated glycosaminoglycan oligosaccharides as well as sulphated derivatives.

#### Overloading

Radiolabelled dermatan sulphate and hyaluronic acid oligosaccharides were mixed with increasing concentrations of partially degraded non-radiolabelled chondroitin 4-sulphate and hyaluronic acid respectively, prior to electrophoresis on polyacrylamide gels (Fig. 6). With hyaluronic acid fragments, discrete banding patterns were observed at low concentrations of undegraded material (tracks 9 and 10), but the bands started to merge at a loading of  $42.5\ \mu\text{g}$  of hyaluronic acid (track 8) and a smearing effect was found at the highest loading ( $170\ \mu\text{g}$ , track 6). By contrast, dermatan sulphate oligosaccharides gave good banding patterns even at a chondroitin 4-sulphate loading of  $85\ \mu\text{g}$  (track 2); at the highest loading ( $170\ \mu\text{g}$ , track 1) the large molecular size species were poorly resolved and a 'blurring' effect was observed with the lower- $M_r$  bands. No differences in mobility of individual bands were observed. However, reduced mobilities may arise when high loadings of an individual oligosaccharide are applied to polyacrylamide gels (Cowman *et al.*, 1984).

#### Quantification

Banding patterns produced by fluorographic imaging can be readily quantified by scanning with an LKB Ultrosan Densitometer, model 2202, fixed wavelength. Fig. 7 shows a scan of tracks 8 and 16 of Fig. 1 in which 60 bands have been identified and quantified.

#### Experimental variables

Tris/borate and Tris/glycine buffers gave identical banding patterns. The presence of  $8\text{M}$ -urea in buffers used for resolving dermatan sulphate oligosaccharides did not unduly influence the electrophoretic properties. This is an important consideration in view of the tendency of some glycosaminoglycans to self-associate in non-denaturing conditions (Fransson *et al.*, 1981); if this were to occur during electrophoresis, migration velocities would change with consequent errors in identification of the bands. For best resolution of high- $M_r$  oligosaccharides relatively low voltages ( $500\text{V}$ ) and long running times are recommended, whereas improved separation of the smaller species is achieved at high voltages (up to  $1\text{kV}$ ) and short electrophoresis periods ( $1\text{--}1.5\text{h}$ ). A useful approach when a wide range of oligosaccharide sizes are found in an individual sample is to make two or more loadings at different times onto the same gel.

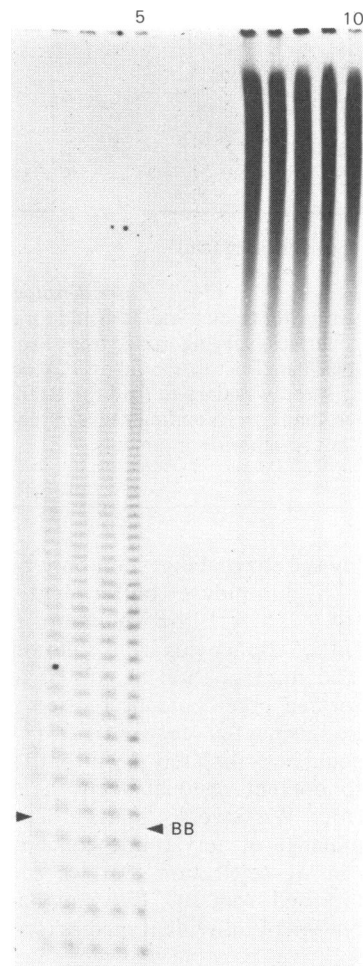


Fig. 6. Influence of sample concentration on the electrophoretic mobility of glycosaminoglycan oligosaccharides. Aliquots ( $10\ \mu\text{l}$ ) of radiolabelled dermatan sulphate oligosaccharides were applied to the gel with varying amounts of chondroitin 4-sulphate partially degraded with chondroitin AC lyase; tracks 1–5 contained  $170\ \mu\text{g}$ ,  $85\ \mu\text{g}$ ,  $42.5\ \mu\text{g}$ ,  $8.5\ \mu\text{g}$  and  $0.85\ \mu\text{g}$  respectively of the glycosaminoglycan. Tracks 6–10 contained, in order, an identical series of decreasing loadings of hyaluronic acid partially degraded with testicular hyaluronidase together with [ $^3\text{H}$ ]oligosaccharides derived from similar enzyme treatments of [ $^3\text{H}$ ]hyaluronic acid. Each track contained  $8 \times 10^4$  c.p.m. Electrophoresis was performed in Tris/glycine/EDTA buffer, pH 8.9, at  $600\text{V}$  and  $30\text{mA}$  for  $2.5\text{h}$  and bands were visualized by fluorography.

The first sample loads are then run at low voltages and later loadings at higher voltages. The tracks may be subsequently cross-indexed by the visual marker dye positions. Sample separation was not

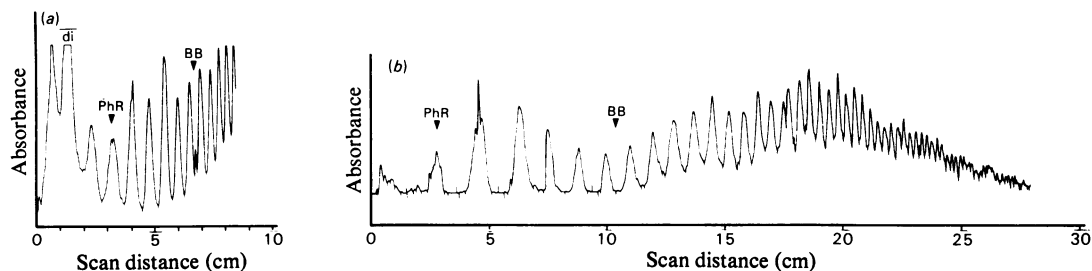


Fig. 7. Laser densitometric scan (a) track 16 and (b) track 8 from Fig. 1

Running time for track 8 was 3.5 h and for track 16 was 1.5 h. Track 16 (a) was used for reading the smaller range of oligosaccharides (varying in size from 1 to 15 disaccharide units in length). The higher- $M_r$  species were read from track 8 but there is a substantial overlap between the two scans as indicated by the dye marker positions. A slower scanning speed was used to read the bands in track 8. Consequently, low- $M_r$  bands in this scan are broader, but shallower, than corresponding peaks in the faster scan of track 16. The major peak, labelled 'di' in (a), contains both di- and hexasaccharide fragments.

affected by the enzyme digestion buffers used (the testicular hyaluronidase buffer had an ionic strength of 0.25). A 1-litre volume of the glacial acetic acid/2,5-diphenyloxazole solution used for fixation and impregnation of gels of the dimensions described here could be re-used four times without impairment of sensitivity. It is of note that the gel should be completely dry prior to autoradiography to prevent residual acetic acid reacting with the film. We have not made a detailed study of the influence of acrylamide concentration on oligosaccharide separation, but we can confirm that gels formed from 10% (w/v) acrylamide give good resolution of individual species (see Cowman *et al.*, 1984). It is possible that more concentrated gels could improve the separation of di- and hexasaccharide fragments of dermatan sulphate (see Fig. 2). Unsaturated dermatan sulphate oligosaccharides produced by chondroitin AC lyase had electrophoretic mobilities similar to those of their unsaturated counterparts produced by testicular hyaluronidase. Charge density is an important factor in determining the mobility of glycans of equivalent carbohydrate content. For example, the hyaluronate disaccharide only has the mobility of a nine-disaccharide unit from dermatan sulphate (I. N. Hampson & J. T. Gallagher, unpublished work). Increases in the degree of sulphation of di- and tetra-saccharides may account for the minor bands seen moving ahead of the main tetrasaccharide band in Fig. 1, tracks 9–13. The influence of the frequency and the position of sulphate residues will be important considerations if polyacrylamide-gel electrophoresis is used in studies of the sulphation process during glycosaminoglycan biosynthesis. The method described in this paper is essentially analytical: that described by Cowman *et al.* (1984) in the accompany-

ing paper employs thicker gels and could be adapted for preparative use.

## Discussion

The results shown demonstrate the high resolving power of polyacrylamide-gel electrophoresis for glycosaminoglycan oligosaccharides. Molecular size and charge density are the major, but not exclusive, determinants of electrophoretic mobility. The relatively low mobility of the dermatan sulphate disaccharide (Fig. 2) remains an intriguing and inexplicable observation at the present time. Non-sulphated hyaluronate disaccharides also migrate more slowly than the corresponding tetrasaccharide. Polyacrylamide gels have been used successfully in the sequence analysis of DNA in which oligonucleotides differing in size by a single nucleotide may be separated (Maxam & Gilbert, 1977) and clearly such methods are applicable to the analysis of linear polysaccharides. It should be emphasized that in this study we have used polymer chains of relatively uniform charge density. Disaccharides from hyaluronic acid contain only a single charged residue (a carboxy group of the glucuronic acid constituent) and dermatan sulphate disaccharides contain two charged residues, the carboxy group and the sulphate residue. A small proportion of disulphated disaccharides has been identified in dermatan sulphate and the position of the sulphate residue itself may vary, being located either at the C-4 of GalNAc or, more rarely, at C-2 of IdoA (Malmström *et al.*, 1975). These variations did not appear to influence the banding pattern obtained with dermatan sulphate oligosaccharides. For copolymers of non-uniform charge density such as heparan sulphate, resolution of oligosaccharides



derived by treatment of the glycan chains with nitrous acid has not been achieved so far (Fig. 2).

Testicular hyaluronidase may catalyse transglycosylations between the reducing GalNAc and the non-reducing GlcA residues of separate oligosaccharide fragments (Weissman, 1955; Highsmith *et al.*, 1975). Such effects would not be detected in the oligosaccharide banding patterns on polyacrylamide gels since the glycans formed would have the same general formula as the primary products of testicular hyaluronidase degradation of dermatan sulphate (see the Results section). Transglycosylation might be controlled by including  $\beta$ -glucuronidase in the digestion mixture.

Some of the potential applications of the new method are demonstrated in this paper. The  $M_r$  values and heterogeneity of native glycosaminoglycan chains may be accurately and rapidly determined. The full extent of the structural heterogeneity of copolymeric dermatan sulphate is clearly defined by the banding pattern shown in Fig. 2. This in turn reflects the extensive glucuronate-to-iduronate transformation at the polymer level. Polyacrylamide-gel systems should allow detailed kinetic and structural analyses of the epimerization process and will help to identify factors which determine the rate and extent of this biologically significant modification step. As the method may be used preparatively (Cowman *et al.*, 1984) relatively high- $M_r$  oligosaccharides of precisely defined size and structure may be obtained for analyses of their conformation and biological activity. Many proteins, such as fibronectin, collagen, laminin and antithrombin III, interact specifically with glycosaminoglycan chains (Lindahl & Höök, 1978; Yamada, 1983; Timpl *et al.*, 1983). The relationships of both the size and sequence of glycosaminoglycan oligosaccharides to their protein-binding properties could be examined in detail by adopting the present methods for affinity electrophoresis. It is widely believed that in their native proteoglycan form glycosaminoglycans fulfil not only important structural roles but may also be involved in the regulation of many cellular processes, particularly since they are widely distributed in cell-surface membranes and in the immediate pericellular matrix (Kraemer, 1979; Gallagher & Hampson, 1984). The considerable heterogeneity of dermatan sulphate chains demonstrated in the present paper may reflect such functional diversity. Methods described here will

be essential to a full understanding of such structure-function inter-relationships of glycosaminoglycans.

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## References

- Cowman, M. K., Slahetka, M. F., Hittner, D. M., Kim, J., Forino, M. & Gadelrab, G. (1984) *Biochem. J.* **221**, 707–716
- Fransson, L.-Å., Havsmark, B. & Sheehan, J. K. (1981) *J. Biol. Chem.* **256**, 13039–13043
- Gallagher, J. T., Gasiunas, N. & Schor, S. (1983a) *Biochem. J.* **215**, 107–116
- Gallagher, J. T., Spooncer, E. & Dexter, T. M. (1983b) *J. Cell Sci.* **63**, 155–171
- Gallagher, J. T. & Hampson, I. N. (1984) *Biochem. Soc. Trans.* **12**, 303
- Hampson, I. & Gallagher, J. T. (1983) *Proc. Int. Symp. Glycoconjugates 7th*, 70 (abstr.)
- Highsmith, S., Garvin, J. H. & Chipman, D. M. (1975) *J. Biol. Chem.* **250**, 7473–7480
- Hiyama, K. & Okada, S. (1975) *J. Biol. Chem.* **250**, 1824–1828
- Kraemer, P. M. (1979) in *Mucopolysaccharides: Cell Biology and Malignancy in Surfaces of Normal and Malignant Cells* (Hynes, R. O., ed.), pp. 149–198, John Wiley and Sons, Chichester and New York
- Lindahl, U. & Höök, M. (1978) *Annu. Rev. Biochem.* **47**, 385–417
- Malmström, A., Carlstedt, I., Aberg, L. & Fransson, L.-Å. (1975) *Biochem. J.* **151**, 477–489
- Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 560–564
- Prehm, P. (1983) *Biochem. J.* **211**, 181–189
- Pulleyblank, D. E. & Booth, G. M. (1981) *J. Biochem. Biophys. Methods* **4**, 339–346
- Riesenfeld, J., Höök, M. & Lindahl, U. (1982) *J. Biol. Chem.* **257**, 421–425
- Rodén, L. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 267–371, Plenum Press, New York
- Sanger, F. & Coulson, A. R. (1975) *J. Mol. Biol.* **94**, 441–448
- Timpl, R., Engel, J. & Martin, G. R. (1983) *Trends Biochem. Sci.* **8**, 207–209
- Weissmann, B. (1955) *J. Biol. Chem.* **216**, 783–794
- Yamada, K. M. (1983) *Annu. Rev. Biochem.* **52**, 761–799