Polyacrylamide-gel electrophoresis and Alcian Blue staining of sulphated glycosaminoglycan oligosaccharides

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Oligosaccharide fragments of glycosaminoglycans may be separated for rapid analysis by electrophoresis through a 10% polyacrylamide matrix. An extensive ladder-like set of bands is observed for partial testicular hyaluronidase digests of chondroitin 4- or 6-sulphate, and for dermatan sulphate. Co-electrophoresis of purified oligosaccharides has established that the major bands of these patterns represent fragments differing in chain length by one disaccharide unit, with the smallest fragments having the greatest mobility. Additional minor bands, representing heterogeneity in the repeating unit structure, are also observed. There are slight differences in the mobilities of oligosaccharides derived from the three major types of sulphated glycosaminoglycans. Alcian Blue is employed for visualization of the digest fragments. Sample loads of 5–10 µg per band appear optimum. The smallest oligosaccharide which may be stained by this method is the hexasaccharide. After consideration of this effect, a good correlation is found to exist between densitometric scans of the gel-electrophoretic patterns and gel-filtration chromatographic profiles based on uronic acid concentration.

The separation of glycosaminoglycan oligosaccharides for purification and analysis is usually performed by the well-established methods of gel filtration and ion-exchange chromatography. By gel-filtration chromatography, oligosaccharides differing in chain length may be separated (Fransson & Rodén, 1967b; Hardingham & Muir, 1973; Hascall & Heinegård, 1974; Christner *et al.*, 1979; Kimura *et al.*, 1979; Cowman *et al.*, 1981). For the sulphated glycosaminoglycans, these oligosaccharides frequently retain heterogeneity in the positions and degree of sulphation, as well as species containing deviations from the idealized repeating structures. Subsequent ion-exchange chromatography can separate oligosaccharides of a given size according to charge density (Fransson, 1968a,b; Malmström & Fransson, 1971; Höök *et al.*, 1974). These methods are the most appropriate techniques available for preparative scale separa-

tions, but are excessively time-consuming for analytical purposes. The newer technique of high pressure liquid chromatography is more rapid, and has the potential to replace earlier techniques in the analysis of small oligosaccharides (Knudsen *et al.*, 1980; Delaney *et al.*, 1980a,b).

An alternative technique which has proven valuable in nucleic acid research is polyacrylamide-gel electrophoresis. Oligo- and polynucleotides, having identical charge-to-mass ratios and similar conformational constraints, are separated according to molecular size by a sieving effect. Particularly striking examples of the high-resolution capabilities of this method can be found in the application of electrophoresis to the determination of nucleic acid restriction fragment sizes (Maniatis *et al.*, 1975), and in the techniques used in the sequencing of DNA fragments (Maxam & Gilbert, 1977). In the latter case, chains containing from less than five to over 100 nucleotides were resolved into discrete bands in a single experiment.

The present work, as well as that in the accompanying paper by Hampson & Gallagher (1984), represents an effort to apply the resolving power of

Abbreviations used: GlcA, β -D-glucopyranuronic acid; IdoA, α -L-idopyranuronic acid; GalNAcS, 2-acetamido-2-deoxy- β -D-galactopyranose 4- or 6-sulphate.

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polyacrylamide-gel electrophoresis to the analysis of glycosaminoglycan fragments. Previous applications of polyacrylamide or agarose electrophoresis to the separation of polymeric glycosaminoglycans yielded rather diffuse bands, resulting from charge density and molecular size heterogeneity (Horner, 1967; Hilborn & Anastasiadis, 1971; Bader *et al.*, 1972; McDuffie, 1981; Nader *et al.*, 1981). The method described herein yields a series of discrete bands for glycosaminoglycan oligosaccharides which, for a given repeating structure, are primarily separated according to size by electrophoresis through a concentrated polyacrylamide matrix. An Alcian Blue staining procedure is employed for detection, allowing the rapid analysis of mixtures of oligosaccharides available in quantities of approx. 1–10 µg for each component species.

It is anticipated that polyacrylamide-gel electrophoresis will prove useful in studies of the kinetics, mechanisms and products of enzymic or chemical cleavages of glycosaminoglycans, in the preparation and characterization of acidic oligosaccharides in general, and in the potential development of rapid methods for sequencing complex carbohydrate structures.

A preliminary account of this work was communicated to the Society for Complex Carbohydrates (U.S.A.), in Lexington, KY, October 1983.

Materials and methods

Materials

Chondroitin 4-sulphate from whale cartilage, chondroitin 6-sulphate from shark cartilage and dermatan sulphate from pig skin were obtained as 'super special grade' materials from Seikagaku Ltd., through Miles Laboratories. All were supplied and used in the sodium salt form. Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35 at 14000 USP/NF units/mg) was obtained from Worthington Biochemical Corp. Chondroitinase AC (chondroitin AC lyase, EC 4.2.2.5, from *Arthrobacter aurescens*) was obtained from Sigma Chemical Co. Gelatin was obtained from Fisher Scientific Co. Bio-Gel P-6 (200–400 mesh) for gel filtration chromatography, Bio-Rad AG 50WX resin for ion exchange, and all reagents for polymerization of electrophoretic gels were obtained from Bio-Rad Laboratories. Alcian Blue 8 GS was obtained from Fluka Chemical Corp. All other chemicals employed were reagent grade quality.

Electrophoresis plates and the vertical slab gel apparatus were obtained from Aquebogue Machine and Repair Shop, Box 205, Main Road, Aquebogue, NY 11931, U.S.A. They were manu-

factured essentially according to the description given by Studier (1973).

Enzymic digestion of glycosaminoglycans

A 100 mg quantity of chondroitin 4-sulphate or 6-sulphate was dissolved overnight at 4°C in 50 ml of 0.15M-NaCl/0.1M-sodium acetate, pH 5.0 (saline/acetate buffer). Gelatin was dissolved in hot saline/acetate at a concentration of 1% (w/v), and stored overnight at 4°C to gel. Hyaluronidase was dissolved immediately prior to use in cold saline/acetate at a concentration of 4500 units/ml.

The chondroitin sulphate and gelatin solutions were preincubated at 37°C prior to digestion. At $t = 0$, 5 ml of gelatin solution and 2.5 ml of hyaluronidase were added to the glycosaminoglycan. Gelatin is added to digest mixtures for which long-term incubations are used (Weissmann *et al.*, 1954; Hoffmann *et al.*, 1956), in an effort to stabilize hyaluronidase present at low concentration. The mixture was incubated at 37°C with occasional stirring. At $t = 0.5, 1, 2$, and 24 h, aliquots (12.5 ml) were removed, boiled for 10 min, and frozen in separate portions for chromatography (11.5 ml) or electrophoresis (1 ml). Additional 1.25 ml aliquots of hyaluronidase were added to the digest after removal of the 1 h and 2 h samples.

For digestion of dermatan sulphate, a 56 mg sample was dissolved in 25 ml of saline/acetate and digested as above, except that enzyme additions of 3400, 3400, and 4500 units were made at $t = 0, 4$ and 48 h, respectively. After a total digestion time of 72 h, the digest was boiled for 10 min. An aliquot of this was frozen for later electrophoresis, and the remainder was prepared for chromatography.

Gel filtration chromatography and oligosaccharide isolation

Frozen digest samples were thawed rapidly, dried by rotary evaporation, and redissolved overnight at 4°C in 6 ml of 0.5M-pyridinium acetate, pH 6.5. The samples were chromatographed on a column (2.5 cm × 195 cm) of Bio-Gel P-6 at a flow rate of 28 ml/h. Fractions (7 ml) were collected and analysed for uronic acid concentration by using the automated carbazole method (Balazs *et al.*, 1965). Appropriate fractions were pooled to yield purified oligosaccharide samples containing from two to seven disaccharide repeat units. The oligosaccharides were dried, freed of pyridine, and converted to the Na⁺ salt as previously described for hyaluronate oligosaccharides (Cowman *et al.*, 1981, 1983).

The gel-filtration chromatographic method results in almost complete separation of oligosaccharides containing less than eight disaccharide units (see the Results section), so that peak counting and K_{av} values are an appropriate indication of

the number of disaccharide repeats in a given species. The smallest oligosaccharide produced in significant quantities by bovine hyaluronidase is the tetrasaccharide (Weissmann *et al.*, 1954; Weissmann, 1955; Hoffmann *et al.*, 1956). Additional evidence for chain length designations was obtained by quantification of the unsaturated disaccharide produced by chondroitinase AC digestion of the purified oligosaccharides. Approx. 1 mg of oligosaccharide, quantified by uronic acid analysis of a concentrated aqueous solution, was diluted into 1 ml of 0.05 M Tris/0.06 M-sodium acetate/0.05 M-NaCl containing bovine serum albumin (0.1 mg/ml), at pH 7.5, and preincubated at 37°C for 0.5 h. A unit (as defined by Yamagata *et al.*, 1958) of chondroitinase AC, dissolved at 5 units/ml in the same buffer, was added to each sample, and the digest proceeded at 37°C for 20 h. The digests were then boiled for 10 min. Samples were diluted with 8 ml of 0.05 M-KH₂PO₄, pH 1.5, and water was added to bring the final volume to 10 ml. The absorbance at 232 nm was measured, corrected for the weak absorbance of saturated disaccharide, and used to calculate the concentration of unsaturated disaccharide liberated, using ϵ_{232} 5500 litre·mol⁻¹·cm⁻¹ for chondroitin 6-sulphate and 5100 litre·mol⁻¹·cm⁻¹ for chondroitin 4-sulphate (Yamagata *et al.*, 1968). The molar ratios of unsaturated disaccharide to total initial uronic acid for chondroitin 6-sulphate species were 0.51 (theory 0.50) for the tetrasaccharide, 0.70 (theory 0.67) for the hexasaccharide, 0.75 (theory 0.75) for the octasaccharide, and 0.82 (theory 0.80) for the decasaccharide. The chondroitin 4-sulphate tetrasaccharide yielded a value of 0.53 (theory 0.50). Other oligosaccharide sizes were inferred from K_{av} values and peak counting.

Oligosaccharides were stored frozen in aqueous solution at 1 mg/ml.

Polyacrylamide-gel electrophoresis

The electrophoretic method was adapted from that described by Maniatis *et al.* (1975), using a buffer system similar to that of Peacock & Dingman (1967). The acrylamide stock solution, stored protected from light at 4°C, contained 20% (w/v) acrylamide and 0.67% *NN'*-methylenebisacrylamide. The concentrated buffer stock, stored at 4°C, contained 0.2 M-Tris/borate/0.002 M-disodium EDTA, pH 8.3 (2 × Tris/borate/EDTA). Equal volumes of these two solutions were mixed at room temperature and deaerated for 15 min. For polymerization, 0.4 ml of 10% (w/v) ammonium persulphate in water (freshly prepared) and 0.04 ml of *NNN'N'*-tetramethylethylenediamine were added per 100 ml of gel solution. The gels (15 cm long and 14 cm wide) were cast between glass plates spaced by perspex strips of 0.25 cm thickness.

Sample wells 1.5 cm deep and 1.1 cm wide were formed by a perspex 'comb' having eight teeth. The gels were overlaid with water and allowed to polymerize overnight at room temperature before use.

The concentrations of oligosaccharide samples were determined by automated uronic acid analysis (Balazs *et al.*, 1965). Digest sample concentrations were calculated from the initial weight of polymer in the digest mixture. For electrophoresis, samples containing 5–100 µg of material in less than 50 µl volume were mixed with a one-tenth volume of 2 M-sucrose in 1 × Tris/borate/EDTA buffer (a 1:1 dilution of the stock), and applied to the gel. Bromphenol Blue (0.2% in 2 M-sucrose in 1 × Tris/borate/EDTA) was used as a tracking dye, but was generally applied to a well with no sample, since its mobility matched that of a chondroitin sulphate oligosaccharide with approx. 13 repeating units. The reservoir buffer was 1 × Tris/borate/EDTA. Gels were electrophoresed at 20 mA (125 V) for 20 min and then at 40 mA (250 V) for approx. 1.5 h. The gels were stained in 0.5% Alcian Blue in 2% acetic acid for 45 min, then destained for 15 min in 2% acetic acid. For quantification of the stained pattern, gels were scanned at 615 nm on a Kontes model 800 scanning densitometer.

Results

Polyacrylamide-gel electrophoresis of chondroitin sulphate digests

Partial cleavage of chondroitin 4-sulphate or 6-sulphate with bovine testicular hyaluronidase (Meyer & Rapport, 1950; Mathews *et al.*, 1951) yields a series of oligosaccharide fragments with the predominant repeating structures (GlcA-GalNAcS)_n. In the present work, an electrophoretic technique for the analysis of the oligosaccharide mixtures is described.

Fig. 1 shows the pattern of stained bands observed after polyacrylamide-gel electrophoresis of chondroitin sulphate samples digested for increasing periods of time with bovine testicular hyaluronidase. The patterns obtained for chondroitin 4-sulphate and chondroitin 6-sulphate are similar. In both cases, a ladder-like set of bands is found at the early time point of digestion. With increasing exposure to enzyme, the intensity of the bands with the greatest mobility increases, concomitant with the disappearance of the slower moving bands. The electrophoretic mobility of a given band is not affected by the extent of digestion. The apparent reduction in mobility near the sides of the gel is due to uneven heating in the gel slab during electrophoresis (see below).

In polyacrylamide-gel electrophoresis of DNA fragments or protein-detergent complexes, species

Fig. 1. Polyacrylamide-gel electrophoresis of chondroitin 6-sulphate (lanes 1-4) and chondroitin 4-sulphate (lanes 5-8) digested with bovine testicular hyaluronidase for increasing periods of time

Digestion conditions were as described in the text. Digest aliquots were mixed with sucrose in Tris/borate/EDTA buffer and electrophoresed through a 10% polyacrylamide gel. The gel was stained with 0.5% Alcian Blue in 2% acetic acid. Digestion time periods and sample loads applied to the gel were as follows: lanes 1 and 8, 24 h, 30 μ g; lanes 2 and 7, 2 h, 40 μ g; lanes 3 and 6, 1 h, 50 μ g; lanes 4 and 5, 0.5 h, 50 μ g. The Bromphenol Blue tracking dye was added to lane 1. It is a faint band, with a mobility approximately two-thirds of that for the major sample band (hexasaccharide) in the same lane.

of approximately equal charge-to-mass ratios and conformational features are separated primarily on the basis of size, with the lowest M_r species having the greatest mobility through the polyacrylamide matrix. The changes in the patterns of glycosaminoglycan fragments observed as a function of the digestion conditions suggest that this mode of separation also occurs in the present case.

The individual bands in the stained gel were identified by coelectrophoresis of purified oligosaccharides. Oligosaccharides ranging in size from the tetrasaccharide ($n = 2$) to the tetradecasaccharide ($n = 7$) were isolated from digests by gel filtration chromatography (Figs. 2 and 3). The electrophoretic results for chondroitin 6-sulphate fragments are shown in Fig. 4. Each oligosac-

Fig. 2. Analysis of chondroitin 6-sulphate oligosaccharides produced by testicular hyaluronidase digestion: comparison of profiles obtained by gel filtration chromatography on Bio Gel P-6 with densitometric scans of gel electrophoretic patterns on 10% polyacrylamide

Chondroitin 6-sulphate was digested with bovine testicular hyaluronidase as described in the text, and aliquots were removed at 1 h (top), 2 h (middle) and 24 h (bottom). The peak numbers correspond to the number of repeating disaccharide units in each oligosaccharide. In the electrophoretic profiles, 3a is the minor hexasaccharide component, and X is the band tentatively ascribed to a minor tetrasaccharide component. Relative staining capabilities for these species have not been established. Small differences in the gel filtration elution position of the oligosaccharides in separate chromatograms are due to pressure-induced alterations of the column bed volume.

charide yielded one major stained band, whose mobility matched that of a corresponding band in the original digest mixture. The digest mixture bands therefore arise from discrete oligosaccharide species differing in chain length by one disaccharide unit.

A plot of mobility versus log oligosaccharide M_r is linear for the larger species ($4 < n < 20$), as has previously been observed for DNA fragments (Maniatis *et al.*, 1975). Smaller oligosaccharides move slightly less rapidly than expected.

The purified oligosaccharides also show a slight apparent heterogeneity, which for the larger oligosaccharides is manifested as a diffusely stained region migrating ahead of the main component. These regions are tentatively assigned to partially

oversulphated material, with a greater charge-to-mass ratio, since shark cartilage chondroitin 6-sulphate is known to contain oversulphated residues (Seno *et al.*, 1975). For the larger oligosaccharides, oversulphation of one or two residues causes a small change in the total charge-to-mass ratio. For smaller oligosaccharides, the effect is more marked. The octasaccharide ($n = 4$) of chondroitin 6-sulphate shows one stained band at the position expected, and a second lighter band of greater mobility. This second band is not the result of contamination with the hexasaccharide, since rechromatography of the isolated octasaccharide on Bio-Gel P-6 showed a single symmetrical peak at the expected position. The purified hexasaccharide similarly shows two discrete bands on

Fig. 3. Analysis of chondroitin 4-sulphate oligosaccharides produced by testicular hyaluronidase digestion: comparison of profiles obtained by gel filtration chromatography on Bio-Gel P-6 with densitometric scans of gel electrophoretic patterns on 10% polyacrylamide

Chondroitin 4-sulphate was digested with bovine testicular hyaluronidase as described in the text, and aliquots were removed at 0.5 h (top), 2 h (middle) and 24 h (bottom). The peak numbers correspond to the number of repeating disaccharide units in each oligosaccharide.

electrophoresis, but contains no tetrasaccharide contamination. Pure tetrasaccharide fails to stain under the conditions used. The band tentatively ascribed to oversulphated hexasaccharide in the chondroitin 6-sulphate pattern is extremely faint in the chondroitin 4-sulphate digest pattern.

In addition to the major bands identified as specific oligosaccharide species differing in molecular size, and the minor bands attributed to oversulphated species, there is one faster band in the chondroitin 6-sulphate digest patterns. Its identity is not yet known, but it may correspond to an oversulphated tetrasaccharide. A very faint band appears at this position for heavy loads (40–80 µg) of purified chondroitin 6-sulphate tetrasaccharide. Purified chondroitin 4-sulphate tetrasaccharide failed to stain at a load of 80 µg.

The maximum number of stained bands generally visible in a 10% polyacrylamide gel of 15 cm length is approx. 30, under conditions which result in migration of the hexasaccharide to the end of the

gel. The undigested chondroitin sulphate polymers show little or no material in this region, but move as diffuse bands with heaviest staining approx. 1.5 cm from the origin, and lighter staining extending nearly to the $n = 30$ band. A similar analysis of undigested dermatan sulphate showed a lower degree of heterogeneity, with a relatively narrow band located at a position calculated to represent 40 ± 10 disaccharide units of dermatan sulphate (see below).

Structural dependence of electrophoretic mobility

The mobilities of sulphated oligosaccharides of equal M_r but different covalent structures are not identical. Examination of the early digest patterns in Fig. 5 shows that the chondroitin 4-sulphate and chondroitin 6-sulphate bands are slightly and progressively offset, with the lower- M_r bands of chondroitin 4-sulphate showing a greater mobility than those of chondroitin 6-sulphate. This difference in mobility is also seen in a direct comparison of the

Fig. 4. Identification of bands in the electrophoretic profile of a chondroitin 6-sulphate digest: co-electrophoresis of chondroitin 6-sulphate oligosaccharides purified by chromatography on Bio-Gel P-6

Electrophoresis was on 10% polyacrylamide, stained with 0.5% Alcian Blue in 2% acetic acid. Lanes 2, 4, and 6, chondroitin 6-sulphate, digested for 0.5 h with bovine testicular hyaluronidase, sample load 50 μ g; lane 1, tetradecasaccharide ($n = 7$) and hexasaccharide ($n = 3$), 10 μ g each; lane 3, dodecasaccharide ($n = 6$), 10 μ g; lane 5, decasaccharide ($n = 5$), 10 μ g; lane 7, octasaccharide ($n = 4$), 10 μ g; lane 8, Bromphenol Blue tracking dye.

purified tetradecasaccharides ($n = 7$) of each type. The chondroitin 4-sulphate species migrates approx. 1% faster than the chondroitin 6-sulphate fragment of the same chain length.

Dermatan sulphate oligosaccharides were also compared to the chondroitin sulphate fragments. Digestion of pig skin dermatan sulphate with bovine testicular hyaluronidase cleaves hexosaminidic linkages to glucuronate residues, yielding a variety of oligosaccharide species (Fransson & Rodén, 1967*a,b*; Fransson & Malmström, 1971). The oligosaccharide pattern obtained after a long-term digestion is shown in Fig. 5. The low- M_r dermatan sulphate fragments migrated approx. 1% more slowly than chondroitin 6-sulphate oligosaccharides of the same M_r , and approx. 2% more slowly than those of chondroitin 4-sulphate. This was again confirmed by a direct comparison of the purified tetradecasaccharides.

The small difference in migration rates of the chondroitin 4-sulphate and chondroitin 6-sulphate oligosaccharides results in apparent synchronization of the bands for the higher- M_r species. The bands for the $n = 15$ fragments are approximately matched in migration distance. For dermatan sulphate fragments, however, the higher- M_r species migrate significantly more slowly than expected. The band corresponding to $n = 14$ of dermatan sulphate matches the band for $n = 15$ of chondroitin

4- or 6-sulphate. This apparent anomaly may in part derive from the unique structure of the dermatan sulphate oligosaccharides. In small dermatan sulphate fragments, the nonreducing terminal GlcA comprises a significant fraction of the total uronic acid. Higher- M_r dermatan sulphate fragments contain predominantly (but not exclusively) IdoA. Thus the smallest species of dermatan sulphate might be expected to resemble the purely GlcA-containing chondroitin sulphates more closely.

Charge-to-mass ratios also affect oligosaccharide mobility. Oligosaccharides derived from hyaluronate and chondroitin migrate much less rapidly than the sulphated oligosaccharides already discussed. A hyaluronate fragment containing 10 repeating disaccharide units has approximately the same mobility as a chondroitin 6-sulphate fragment of 19 disaccharide units. Details of the electrophoretic and staining characteristics of the nonsulphated glycosaminoglycan fragments will be described elsewhere (M. K. Cowman & R. E. Turner, unpublished work).

Alcian Blue staining of chondroitin sulphate oligosaccharide

Alcian Blue, a copper phthalocyanine derivative with multiple cationic substituent groups (Scott *et al.*, 1964), is a sensitive stain for the acidic glycos-

Fig. 5. Comparison of electrophoretic mobilities of oligosaccharides from chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate

Electrophoresis was on 10% polyacrylamide, stained with 0.5% Alcian Blue in 2% acetic acid. Sample loads were 50 μ g for the unfractionated digest mixtures, and 10 μ g for purified oligosaccharides. Lanes 1 and 8, Bromphenol Blue tracking dye; lane 2, chondroitin 6-sulphate, digested for 0.5 h with bovine testicular hyaluronidase; lane 3, chondroitin 4-sulphate, digested for 0.5 h with bovine testicular hyaluronidase; lane 4, dermatan sulphate, digested for 72 h with bovine testicular hyaluronidase; lane 5, tetradecasaccharide from chondroitin 6-sulphate; lane 6, tetradecasaccharide from chondroitin 4-sulphate; lane 7, tetradecasaccharide from dermatan sulphate.

aminoglycans (Quintarelli *et al.*, 1964). Alcian Blue appears to precipitate and immobilize glycosaminoglycan oligosaccharides within the polyacrylamide matrix, and therefore serves as both fixative and stain. Gels show some diffusion of dye with time, but retain fairly discrete bands during storage periods of at least a few weeks.

Optimum sample loads were found to be in the range of 5–10 μ g per oligosaccharide band. Lower sample loads (1–3 μ g) are visible, but less easily photographed or scanned.

Further considerations are the chain length dependence and linearity of stain incorporation with increasing load. Some chain length dependence in staining was observed. The tetrasaccharide of chondroitin 4-sulphate did not stain even at high load levels. The weak band observed for the chondroitin 6-sulphate tetrasaccharide at high sample load is attributed to an oversulphated species, on the basis of its high mobility. The hexasaccharide and octasaccharide of chondroitin 6-sulphate may also show slightly diminished staining relative to higher oligosaccharides (Fig. 4), although the existence of multiple bands makes a direct comparison difficult. In order to establish better the relationship between the electrophoretic patterns and the

true composition of an oligosaccharide mixture, the stained gels of digests were scanned at 615 nm with a Kontes model 800 densitometer (Figs. 2 and 3). Excluding the anomalous behaviour of the tetrasaccharide, the gel filtration chromatographic profiles and the electrophoretic patterns were found to be quite similar. The major discrepancies are attributed to the charge heterogeneity of the oligosaccharides. An additional source of error in the present case was found to be a slow continuing digestion of the earliest time point samples, upon repeated thawing for electrophoretic study. In general, the electrophoretic patterns provide a good indication of the oligosaccharide size distribution in the complex digest mixtures.

Experimental considerations

Several experimental factors have been investigated to discern the sensitivity of the method to potential artefacts. The presence of enzyme, NaCl, and sodium acetate in the digest mixtures had little influence on mobility, as judged by the similarity in mobility of purified oligosaccharides and digest bands. Furthermore, addition of NaCl, to a concentration of 0.15 M, to purified oligosaccharides did not affect mobility. The total sample load, how-

ever, has an effect on migration rate. Increasing the sample load of a pure oligosaccharide from 5 to 35 μg causes a measurable reduction in the rate of migration. Similarly, digest samples containing more than 5–10 μg of sample per band show reduced mobility of the overloaded bands. An additional source of changes in mobility is uneven heating of the gel during electrophoresis. In an uncooled apparatus, sample lanes near the edges show a reduced rate of migration. A commercial apparatus which employs buffer immersion of the gel slab eliminates this effect.

Discussion

By polyacrylamide-gel electrophoresis, oligosaccharides derived from a given sulphated glycosaminoglycan are separated primarily according to chain length. Although the largest purified oligosaccharide employed in band identification in the present work contained only seven repeating units, we extrapolate the assignments of slower bands to higher- M_r species, increasing in chain length by one disaccharide unit for each band. With that assumption, band mobility is an approximately linear function of the log of the fragment M_r . This interpretation is supported by the observations of Hampson & Gallagher (1984), which show a clear correlation between the gel filtration elution positions and the electrophoretic mobilities of various high- M_r fragments. Hilborn & Anastasiadis (1971) have previously observed a correlation between M_r and mobility on a 6% polyacrylamide gel for polymeric glycosaminoglycans.

Charge density also influences electrophoretic mobility. Sulphated glycosaminoglycans migrate more rapidly than the nonsulphated species. The observation of multiple bands for the smallest purified chondroitin 6-sulphate oligosaccharides may be due to this effect. Although glycopeptides containing neutral sugars are known to co-purify with glycosaminoglycan oligosaccharides (Fransson, 1968b), the lower charge density of such species makes them an unlikely source of the extra bands. The dermatan sulphate digest similarly shows a faint rapidly migrating band at the same position as the minor hexasaccharide species of chondroitin 6-sulphate. The chondroitin 4-sulphate digests showed only a very faint band in this region, which may indicate a lower degree of oversulphation in the sample employed.

Configurational differences among the sulphated glycosaminoglycans are correlated with small differences in electrophoretic mobility. The basis for this effect is not clear. It is possible that the isomeric glycosaminoglycans differ slightly in effective charge density, as a result of varying tendencies to bind counterions or interact with the

buffer employed. Alternatively, it is possible that the oligosaccharide mobilities reflect conformational differences. In the electrophoresis of linear DNA fragments, certain species exhibit mobilities that are inconsistent with their known molecular weights (Maniatis *et al.*, 1975). It has been speculated that this phenomenon is related to unusual base compositions or sequences, which result in altered conformations or changes in chain flexibility. Similarly, the different mobilities of chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate fragments of identical M_r may reflect subtle differences in chain conformation and/or flexibility. Whatever the basis for the differences in mobility, it should be noted that analysis of mixtures of glycosaminoglycans is likely to be a more complex problem, and that appropriate M_r markers must be used in the identification of fragment sizes.

The enzyme employed in degradation of polymeric glycosaminoglycans is an additional important consideration. Testicular hyaluronidase catalyses transglycosylation by an unknown mechanism (Weissmann *et al.*, 1954; Weissmann, 1955; Hoffmann *et al.*, 1956). Attempts to determine glycosaminoglycan sequences via electrophoretic analysis of the oligosaccharide fragments generated should employ other non-reversible methods and conditions of cleavage.

The electrophoretic separation and staining procedure described in this report provides a facile and rapid assay of the composition of complex oligosaccharide mixtures. It is suitable for modest quantities of unlabelled material containing approx. 1–10 μg of each component species. Longer gels with a higher percentage of polyacrylamide, as employed by Hampson & Gallagher (1984), can provide greater resolution of the higher- M_r components. Additionally, the use of radiolabelled samples allows detection of smaller quantities. The two methods are complementary, in that the gel composition, length, and detection method should be determined by the application at hand.

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References

- Bader, J. P., Ray, D. A. & Steck, T. L. (1972) *Biochim. Biophys. Acta* **264**, 73–84
- Balazs, E. A., Berntsen, K. O., Karossa, J. & Swann, D. A. (1965) *Anal. Biochem.* **12**, 547–558
- Christner, J. E., Brown, M. L. & Dziewiatkowski, D. D. (1979) *J. Biol. Chem.* **254**, 4624–4630
- Cowman, M. K., Balazs, E. A., Bergmann, C. W. & Meyer, K. (1981) *Biochemistry* **20**, 1379–1385
- Cowman, M. K., Bush, C. A. & Balazs, E. A. (1983) *Biopolymers* **22**, 1319–1334
- Delaney, S. R., Leger, M. & Conrad, H. E. (1980a) *Anal. Biochem.* **106**, 253–261
- Delaney, S. R., Conrad, H. E. & Glaser, J. H. (1980b) *Anal. Biochem.* **108**, 25–34
- Fransson, L.-Å. (1968a) *J. Biol. Chem.* **243**, 1504–1510
- Fransson, L.-Å. (1968b) *Biochim. Biophys. Acta* **156**, 311–316
- Fransson, L.-Å. & Malmström, A. (1971) *Eur. J. Biochem.* **18**, 422–430
- Fransson, L.-Å. & Rodén, L. (1967a) *J. Biol. Chem.* **242**, 4161–4169
- Fransson, L.-Å. & Rodén, L. (1967b) *J. Biol. Chem.* **242**, 4170–4175
- Hampson, I. N. & Gallagher, J. T. (1984) *Biochem. J.* **221**, 697–705
- Hardingham, T. E. & Muir, H. (1973) *Biochem. J.* **135**, 905–908
- Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4242–4249
- Hilborn, J. C. & Anastassiadis, P. A. (1971) *Anal. Biochem.* **39**, 88–92
- Hoffmann, P., Meyer, K. & Linker, A. (1956) *J. Biol. Chem.* **219**, 653–663
- Höök, M., Lindahl, U. & Iverius, P.-H. (1974) *Biochem. J.* **137**, 33–43
- Horner, A. A. (1967) *Can. J. Biochem.* **45**, 1009–1013
- Kimura, J. H., Hardingham, T. E., Hascall, V. C. & Solursh, M. (1979) *J. Biol. Chem.* **254**, 2600–2609
- Knudsen, P. J., Eriksen, P. B., Fenger, M. & Florentz, K. (1980) *J. Chromatogr.* **187**, 373–379
- Malmström, A. & Fransson, L.-Å. (1971) *Eur. J. Biochem.* **18**, 431–435
- Maniatis, T., Jeffrey, A. & van deSande, H. (1975) *Biochemistry* **14**, 3787–3794
- Mathews, M. B., Roseman, S. & Dorfman, A. (1951) *J. Biol. Chem.* **188**, 327–334
- Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 560–564
- McDuffie, N. M. (1981) in *Chemistry and Biology of Heparin* (Lundblad, R. L., Brown, W. V., Mann, K. G. & Roberts, H. R., eds.), pp. 57–64, Elsevier/North Holland, Amsterdam
- Meyer, K. & Rapport, M. M. (1950) *Arch. Biochem.* **27**, 287–293
- Nader, H. B., Takahashi, H. K., Guimaraes, J. A., Dietrich, C. P., Bianchini, P. & Ozima, B. (1981) *Int. J. Biol. Macromol.* **3**, 356–360
- Peacock, A. C. & Dingman, C. W. (1967) *Biochemistry* **6**, 1818–1827
- Quintarelli, G., Scott, J. E. & Dellovo, M. C. (1964) *Histochemie* **4**, 86–98
- Scott, J. E., Quintarelli, G. & Dellovo, M. C. (1964) *Histochemie* **4**, 73–85
- Seno, N., Anno, K., Yaegashi, Y. & Okuyama, T. (1975) *Connect. Tissue Res.* **3**, 87–96
- Studier, F. W. (1973) *J. Mol. Biol.* **79**, 237–248
- Weissmann, B. (1955) *J. Biol. Chem.* **216**, 783–794
- Weissmann, B., Meyer, K., Sampson, P. & Linker, A. (1954) *J. Biol. Chem.* **208**, 417–429
- Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535