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Preparation and Circular Dichroism Analysis of Sodium Hyaluronate Oligosaccharides and Chondroitin†

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ABSTRACT: Sodium hyaluronate was cleaved into an homologous series of oligosaccharides by the action of bovine testicular hyaluronidase (EC 3.2.1.35), an endo-β-hexosaminidase. Digestion conditions and gel-filtration chromatographic fractionation were optimized to produce oligosaccharides, clearly separable into peaks corresponding to 1–23 disaccharide units of the type D-glucuronosyl→*N*-acetyl-D-glucosamine. The chromatographic method was also employed for the purification of a second homologous group of oligosaccharides, with the reversed sequence of monosaccharide units, produced by the action of leech hyaluronidase (EC 3.2.1.36), an endo-β-glucuronidase. Circular dichroism (CD) analysis (in the 200–250-nm range) of the oligosaccharides showed that the CD spectrum of hyaluronate in aqueous solution at neutral pH does not reflect to any substantial degree a polymer conformation which requires cooperative interaction between several repeating residues for stabilization. The enhanced CD properties of hyaluronate relative to those of monosaccharides are primarily related to the existence of the β-1,4 linkage from *N*-acetyl-D-glucosamine to D-glucuronate. Chondroitin, the *N*-acetyl-D-galactosamine analogue of hyaluronate, was prepared by chemical desulfation of chondroitin 4- and 6-sulfates. The purified product had a molecular weight range of 4000–8000 (10–20 disaccharide units). It was digested with testicular hyaluronidase, and the split products were isolated by gel filtration. In contrast to hyaluronate, the cleavage products included both the preponderant analogous repeating disaccharide multiples with *N*-acetyl-D-galactosamine at the reducing end and smaller quantities of oligosaccharides composed of an odd number of monosaccharides. These latter products were presumably derived from the ends of the shortened chondroitin chains, resulting from methanolysis during the desulfation and alkaline elimination of reducing hexosamines. CD spectroscopic analysis of chondroitin relative to its constituent monosaccharides showed that this glycosaminoglycan does not share the structural feature which results in substantially enhanced CD properties for hyaluronate. A hypothesis relating the CD properties of N-acetylated hexosamines in oligo- and polysaccharides to the dihedral angle about the carbon–oxygen bond at C3 may explain the CD dependence on both the hexosaminidic linkage and hexosamine configuration at C4.

Hyaluronic acid is a high molecular weight unbranched glycosaminoglycan which is composed of alternating *N*-acetyl-β-D-glucosamine (GlcNAc)[1] and β-D-glucuronic acid (GlcUA) residues linked at the 1,3 and 1,4 positions, respectively (Meyer, 1958). In hydrated stretched membranes, hyaluronic acid and its simple salts are capable of adopting ordered helical conformations (Atkins & Sheehan, 1972; Atkins et al., 1972; Dea et al., 1973; Winter et al., 1975; Guss et al., 1975; Winter & Arnott, 1977), including a possible double-helical form containing two antiparallel chains (Sheehan et al., 1977). In aqueous solution, hyaluronate behaves as a random coil with some stiffness, occupying a domain 10^3–10^4 times larger than the volume of the chain itself (Balazs, 1958). Periodate oxidation kinetics (Scott & Tigwell, 1978) and nuclear magnetic resonance studies (Darke et al., 1975; Welti et al., 1979; Scott & Heatley, 1979) have provided evidence for some degree of segmental rigidity in the polymer, with a probable equilibrium between rigid and flexible structures. The extremely high viscosity and unique viscoelastic properties of hyaluronate solutions are considered to be evidence for specific intermolecular interactions (Balazs, 1966, 1968; Gibbs et al., 1968; Morris et al., 1980; Welsh et al., 1980). The conformational features of rigid or interacting segments of hyaluronate in solution have not yet been elucidated.

Circular dichroism (CD) spectroscopy is a commonly employed technique for the investigation of macromolecular conformation in solution and may provide insight into the structural details of ordered hyaluronate segments. Above 200 nm, the CD spectrum of sodium hyaluronate (NaHA) contains a negative band centered near 209 nm which is more intense than the corresponding bands for monosaccharides (Stone, 1971; Chakrabarti & Balazs, 1973; Balazs et al., 1977; Buffington et al., 1977). This band reflects the n–π* transitions of two chromophoric groups in NaHA: the acetamido and carboxylate moieties. Studies on the CD properties of uronic acids and acetamido sugars indicate a sensitivity to the orientation of neighboring substituents relative to the chromophoric group (Kabat et al., 1969; Listowsky et al., 1969; Yeh & Bush, 1974; Morris et al., 1975; Aubert et al., 1976; Coduti et al., 1977; Dickinson et al., 1977). These data suggest a possible dependence of NaHA CD on the conformational angles about the glycosidic bonds linking the sugars and therefore on hyaluronate conformation. Any ordered conformation which requires cooperative interaction between several disaccharide residues for stability is of necessity chain length dependent. In synthetic oligopeptide studies, it was found that a minimum chain length was required for formation of helical segments and consequent alteration in optical activity (Goodman et al., 1963). For investigation of the possibility of similar requirements for NaHA, the CD properties of a large number of oligosaccharide fragments of differing sizes

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[1] Abbreviations used: CD, circular dichroism; NaHA, sodium hyaluronate; GlcUA, D-glucuronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GalNAc, 2-acetamido-2-deoxy-D-galactose.

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and sequences were investigated. The systematic study of oligosaccharides also provides information on the relative importance of the two different linkages in NaHA to the conformation and CD properties.

Since the report by Meyer and co-workers (Weissmann et al., 1954; Weissmann, 1955) of the breakdown of hyaluronate by testicular hyaluronidase into an homologous series of oligosaccharides by extensive transglycosylation, the procedure has been utilized by various authors for the production of these oligosaccharides. Gel-permeation chromatography was used previously (Flodin et al., 1964; Hardingham & Muir, 1973; Hascall & Heinegard, 1974; Christner et al., 1979; Kimura et al., 1979) for separation of oligosaccharides of NaHA. A satisfactory yield and purity of the larger oligosaccharides were, however, not achieved. In this paper, procedures of controlled enzymatic digestion and chromatographic isolation of oligosaccharides in high yield and of good purity are presented. In addition, the production and separation of homologous oligosaccharides of NaHA produced by the endo-β-glucuronidase (Linker et al., 1957) of the leech are described.

A structural and spectroscopic study of another nonsulfated glycosaminoglycan, chondroitin, has also been undertaken. This polymer, prepared by chemical desulfation of chondroitin 4- or 6-sulfate, differs from NaHA in its lower molecular weight and opposite configuration at C4 of the hexosamine moiety (Hoffman et al., 1958). The importance of that configurational difference to the adoption of ordered conformation in NaHA could therefore be examined, in view of evidence that the desulfated and sulfated forms of chondroitin do not contain the same degree of segmental rigidity found in NaHA (Mathews & Decker, 1977; Welti et al., 1979). The chondroitin used for CD studies was chemically characterized. Oligosaccharides were obtained by testicular hyaluronidase digestion, which include the predominant repeating disaccharide multiples analogous to those obtained for NaHA as well as products with an odd number of monosaccharide units.

Materials and Methods

Sodium Hyaluronate. High molecular weight rooster comb sodium hyaluronate (NaHA) for circular dichroism studies was obtained from Pharmacia (Piscataway, NJ). This was prepared according to the specifications of Balazs (1979) and contained <0.1% protein and <0.1% sulfated glycosaminoglycan relative to the NaHA content. The sterile, pyrogen free preparation (lot DL 10492) was supplied in syringes, with NaHA at a concentration of 9.7 mg/mL in a solvent containing 145 mM NaCl, 0.34 mM NaH_2PO_4, and 1.5 mM Na_2HPO_4, pH 7.2 ± 0.2. The limiting viscosity number of the NaHA was 3700 mL/g, and the relative viscosity at 9.7 mg/mL was 930 000 cSt. The limiting viscosity number may be used to obtain an estimate of the molecular weight, which is 2.4×10^6, based on the relation $[\eta] = 0.029M^{0.80}$ (Balazs, 1965). Samples of various NaHA concentration and ionic strength were prepared by dilution and dialysis at 4 °C. The NaHA used for enzymatic degradation was prepared according to the same procedures from rooster comb as quoted above (Balazs, 1979). The purified NaHA (protein <1% of the NaHA) was stored as a precipitate under ethanol at 4 °C until used. Analysis of this material dissolved in 0.15 M NaCl gave a limiting viscosity number of 2700 mL/g. Analysis of glucosamine and galactosamine on a Beckman Model 121MB amino acid analyzer showed that the galactosamine content was less than the limit of detection. Prior to use for enzymatic digestion, an aliquot of the ethanol precipitate of the NaHA was partially dried by rotary evaporation and stored under vacuum in the presence of P_2O_5 until completely dry.

Digestion of NaHA by Testicular Hyaluronidase. Samples of 50 mg were dissolved in 5 mL of 150 mM NaCl, 100 mM sodium acetate, and 1.0 mM Na_2EDTA, pH 5.0 (buffer A), with slow stirring for 2 days at 4 °C. Prior to digestion, the hyaluronate solution was equilibrated at 37 °C for 0.5 h. To this solution, 0.5 mL of 1% gelatin in buffer A was added. Purified bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35, 15 000–20 000 IU/mg; Leo-Helsingborg, Sweden) was dissolved immediately prior to use in buffer A at a concentration of 1500 IU/mL.

The digestion was started with the addition of 0.33 mL of hyaluronidase solution (10 IU of hyaluronidase/mg of hyaluronate) and stirred slowly at 37 °C. Two additional 0.33-mL aliquots of enzyme solution were added after 1 and 2 h, respectively. The digestion was terminated by immersion in boiling water for 5 min. The digest was dried by rotary evaporation and redissolved in 2–3 mL of 0.5 M pyridinium acetate, pH 6.5.

Digestion of NaHA by Leech Hyaluronidase. Purified leech head hyaluronidase (hyaluronate 3-glycanohydrolase, EC 3.2.1.36) was obtained from Biomatrix, Inc., Riverdale, NY. A 0.5 mg/mL enzyme solution was prepared in 42 mM citric acid and 116 mM Na_2HPO_4, pH 5.6 (McIlvaine's buffer), immeditely prior to use. Approximately 250 mg of dry NaHA was dissolved in 50 mL of McIlvaine's buffer by slow stirring at 4 °C for 2 days. The NaHA solution was equilibrated at 37 °C for 1 h, and then 2 mL of leech hyaluronidase was added and the solution covered with toluene. After digestion with constant slow stirring at 37 °C for 18 h, the reaction was terminated by placing the sample in a water bath at 100 °C for 5 min. The cooled digest was centrifuged at 15000*g* for 20 min to remove precipitated protein and concentrated to a volume of 1–2 mL by rotary evaporation. After addition of 10 mL of 0.5 M pyridinium acetate, pH 6.5, the sample was again centrifuged as above.

Chromatography of Oligosaccharides. Bio-Gel P-30, –400 mesh (Bio-Rad Laboratories, Richmond, CA), was hydrated in water. The resin was slowly equilibrated with 0.5 M pyridinium acetate, pH 6.5, and a 2.5 × 195 cm column was packed and equilibrated under a hydrostatic pressure of 220 cm at a flow rate of 5.5 mL/h. (Consistent properties of this column could be maintained for at least several weeks by perpetual elution, while interruption of the flow resulted in greatly reduced flow rate and resolution.) The digestion products were eluted on this column at a flow rate of 5.5 mL/h. Fractions of 2.5 mL were collected and screened for uronic acid content. Appropriate fractions were pooled, evaporated to dryness by rotary evaporation, and repeatedly washed and evaporated until retaining no odor of pyridine. Samples were dissolved in 3 mL of water, passed through a 2-mL column of Bio-Rad AG 50W-X8 (Na^+), lyophilized, and stored over P_2O_5. For chemical and spectroscopic analysis, the samples were dissolved in twice-distilled water and stored at 4 °C.

Preparation of Trisaccharide. The trisaccharide GlcUA-GlcNAc-GlcUA was prepared by using the method of Linker et al. (1960) from the tetrasaccharide $(GlcUA\text{-}GlcNAc)_2$ obtained from a long-term digest of NaHA by testicular hyaluronidase. A 1% (w/v) aqueous solution of tetrasaccharide was mixed with an equal volume of saturated $Ca(OH)_2$. The solution was stirred at room temperature for 4 h and neutralized with CO_2. Separation of trisaccharide from the elimination product *N*-acetyldehydroglucosamine was performed by elution on a 2-mL column of Bio-Rad AG 1-X10 (acetate) with 10 mL each of water, 0.25 M NaCl, and 0.5 M NaCl. Fractions were desalted on a 1.5 × 85 cm column of Bio-Gel P-2 (100–200 mesh) in water, pooled, and dried.

Chondroitin. Chondroitin, the desulfated product of chondroitin 4- and 6-sulfates, was prepared according to the method of Hirano et al. (1961) from chondroitin 4-sulfate of human chondrosarcoma (SO_4 = 11.6%) and from chondroitin 6-sulfate of the noncalcified vertebral processes of a shark (SO_4= 19.8%). Both preparations, which were free of glucosamine, were converted to the free acid by ion-exchange chromatography of their 1% aqueous solutions on a 20-mL column of Bio-Rad AG 50W-X8 (H^+) at 4 °C. They were immediately frozen, lyophilized, and stored over P_2O_5 in vacuo. Approximately 1 g of the free acid was shaken at room temperature while suspended in 100 mL of dry freshly distilled methanol with 10 g of Bio-Rad AG 50W-X8 (H^+) and 1 g of dry NaCl for 48 or 66 h. After addition of 500 mL of ice-cold water to the solution and stirring for 24 h at 4 °C, the resin was removed by filtration through a plug of glass wool, and the filtrates were dialyzed in the cold against two changes of water. The retentates were concentrated in a rotary evaporator to approximately 30 mL, and a slight precipitate was removed by centrifugation and lyophilized. A 200-mg aliquot was dissolved in 0.5 M NaCl and eluted on a 2.5 × 195 cm column of Bio-Gel P-60 at a flow rate of 13 mL/h. Fractions of 4 mL were collected and screened for uronic acid. Appropriate peaks were pooled, concentrated, and dialyzed in Spectrapore 3 dialysis tubing against several changes of water. The retentates were lyophilized and stored in vacuo over P_2O_5. Approximately 60% of the material eluted as a single broad peak corresponding in size to 10–20 repeating disaccharide units of NaHA (as determined by the K_{av}).

Solutions of this material did not stain with alcian blue and were not digested by testicular hyaluronidase, indicating esterification of the carboxyl group. It was therefore dissolved at 5 mg/mL in water, an equal volume of 0.1 M NaOH was added, and the solution was left for 1 h at room temperature, neutralized with 1 M HCl, and dialyzed against water. After this treatment, its equivalent weight (titimetric) was 404 (theory 401), it stained with alcian blue, and it was digested with testicular hyaluronidase at a rate similar to that for hyaluronate. When the sample of high molecular weight HA was subjected to the "desulfation" procedure, it behaved toward staining and enzymatic digestion identical with the chondroitin.

Chemical Analysis. Uronic acid concentration was determined in duplicate or triplicate by the automated carbazole procedure (Balazs et al., 1965) and reducing *N*-acetylhexosamine by the Reissig et al. (1955) modification of the Morgan & Elson (1934) method using *N*-acetylglucosamine as the standard. Total hexosamine was determined by a modification of the Elson & Morgan method (1933) after hydrolysis in 4 M HCl for 16 h at 95 °C. Reducing terminal glucuronic acid was determined by analysis before and after reduction with a 100-fold excess of $NaBH_4$ at pH 8.2 and room temperature for 2 h, followed by acidification with glacial acetic acid and repeated evaporation with methanol, and finally by dissolving the residue in a known volume of water for analysis.

Spectroscopic Analysis. CD spectra were recorded at room temperature with a Cary 60 with 6001 CD accessory. The instrument sensitivity setting was 20 or 40 mdeg for full-scale deflection. The scan rate was 2 nm/min, using a pen time constant of 3 s, at a spectral resolution of 1.5 nm. The instrument was calibrated routinely with *d*-10-camphorsulfonic acid as described by Fasman (1978). A 1.00-cm fused quartz optical cell was employed for sample concentrations of 0.015–0.19 mg/mL. For studies of concentration dependence, fused quartz cells with calibrated path lengths of 0.097 and 0.0105 cm were employed. The total absorbance of the sample, solvent, and cell combination was limited to a maximum of 1.5 at the lowest wavelength studied. Molar ellipticity values, in units of deg cm^2 $dmol^{-1}$, were calculated on the basis of the disaccharide residue concentration, using the measured uronic acid concentration. The inherent error of an individual spectrum is approximately ±5%. In general, the reported molar ellipticity values are averages of at least four spectra, obtained for two different preparations of each species (two determinations only for $[GlcUA\text{-}GlcNAc]_8$ and $[GlcNAc\text{-}GlcUA]_{5-7}$). Reported errors are the standard deviation calculated for each data set, approximately ±300 deg cm^{-2} $dmol^{-1}$, unless otherwise indicated.

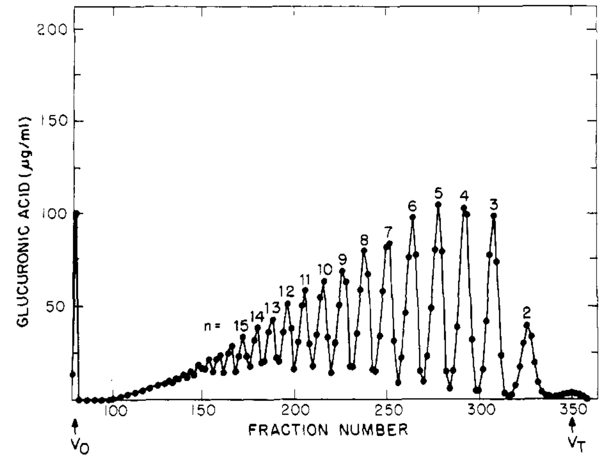

FIGURE 1: Separation of oligosaccharides produced by digestion of NaHA with testicular hyaluronidase. Approximately 50 mg of sample was applied to a column (2.5 × 195 cm) of Bio-Gel P-30, –400 mesh, and eluted with 0.5 M pyridinium acetate, pH 6.5. The number of repeating disaccharide units in each oligosaccharide species is indicated.

Results and Discussion

Preparation and Chemical Analysis of Hyaluronate Oligosaccharides. Degradation of hyaluronate by testicular hyaluronidase, an endo-β-*N*-acetylglucosaminidase, yields an homologous series of oligosaccharides of the type β-D-GlcUA*p*-[(1–3)-β-D-GlcNAc*p*-(1–4)-β-D-GlcUA*p*]$_{n-1}$-(1–3)-D-GlcNAc. In preliminary experiments, the optimum conditions for production and resolution of defined oligosaccharides were investigated. The final results are plotted in Figure 1.

Up to 23 oligosaccharides have been separated. A plot of K_{av} vs. log of peak number fits a normal profile for gel-filtration chromatography (Figure 2); thus the species molecular weight is related to a peak number by a constant factor. By chemical analysis, the ratio of uronic acid to reducing *N*-acetylhexosamine can be determined for the first eight oligosaccharides (Table I); for the larger oligosaccharides, experimental errors preclude accurate determination of chain length by this method. The chromatographic profile shows the regularity of the repeating units.

Since the oligosaccharides are expressions of both hydrolysis and transglycosylation (Weissmann et al., 1954), the polymer appears to contain no units other than multiples of (GlcUA-GlcNAc) and the transglycosylated oligosaccharides form no linkages other than those composing the polymer.

Leech hyaluronidase also produced a regular homologous series of oligosaccharides with the reversed monosaccharide sequence, whose separation was equivalent to that observed

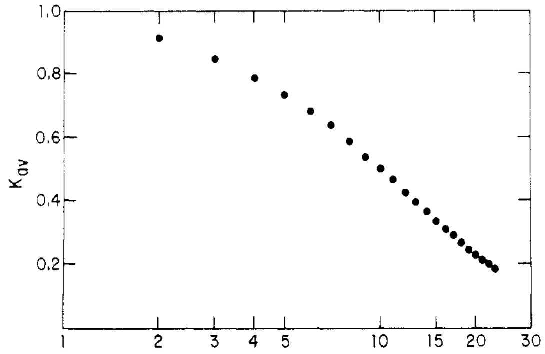

FIGURE 2: Relationship between the elution position (K_{av}) of NaHA oligosaccharide peaks on Bio-Gel P-30 and the peak number, *n*. The peak number is equal to the degree of polymerization for the oligosaccharides $(GlcUA\text{-}GlcNAc)_n$, for $n = 2$–8, by chemical analysis.

Table I: Oligosaccharide Characterization and Relative Size

oligosaccharide	uronic acid/ reducing HexNAc	uronic acid/ total HexN	K_{av}
$(GlcUA\text{-}GlcNAc)_2$	2.00		0.91
$(GlcUA\text{-}GlcNAc)_3$	3.00		0.84
$(GlcUA\text{-}GlcNAc)_4$	4.01		0.78
$(GlcUA\text{-}GlcNAc)_5$	4.99		0.73
$(GlcUA\text{-}GlcNAc)_6$	5.80		0.68
$(GlcUA\text{-}GlcNAc)_7$	6.95		0.63
$(GlcUA\text{-}GlcNAc)_8$	7.76		0.58
GlcUA-GlcNAc-GlcUA	*a*	1.71	
$(GlcNAc\text{-}GlcUA)_2$	*a*	1.07	0.94
$(GlcNAc\text{-}GlcUA)_3$	*a*	0.86	0.89
$(GlcNAc\text{-}GlcUA)_4$	*a*	0.93	0.83
$(GlcNAc\text{-}GlcUA)_5$	*a*	0.98	0.77
$(GlcNAc\text{-}GlcUA)_6$	*a*	0.97	0.71
$(GlcNAc\text{-}GlcUA)_7$	*a*	1.20	0.66

[a] No detectable reducing *N*-acetylhexosamine.

after digestion with testicular hyaluronidase (Table I). The nature of this enzyme as an endo-β-glucuronidase was shown in previous experiments: a hexasaccharide obtained by testicular hyaluronidase digestion, $(GlcUA\text{-}GlcNAc)_3$, upon digestion with leech hyaluronidase yielded two trisaccharides, GlcUA-GlcNAc-GlcUA and GlcNAc-GlcUA-GlcNAc (Linker et al., 1960). It is possible that leech hyaluronidase acts by both hydrolysis and transglycosylation. The chain lengths of the species whose K_{av} correspond to tetrasaccharide and hexasaccharide were established by $NaBH_4$ reduction of terminal GlcUA: the tetraasaccharide $(GlcNAc\text{-}GlcUA)_2$ lost 50% (theory 50%), and the hexasaccharide $(GlcNAc\text{-}GlcUA)_3$ lost 26% (theory 33%) of the total uronic acid.

The trisaccharide GlcUA-GlcNAc-GlcUA was prepared from the tetrasaccharide $(GlcUA\text{-}GlcNAc)_2$. Its purity was established by analysis of the uronic acid to hexosamine ratio (Table I) and by $NaBH_4$ reduction which resulted in a 51% (theory 50%) decrease in total uronic acid.

Preparation and Analysis of Chondroitin. Gel chromatography of the desulfated material on P-60 showed a broad major peak, comprising approximately 60% of the material, and multiple minor peaks of smaller size. Since the major peak contained chondroitin of 4000–8000 molecular weight and the molecular weight of the starting material must have been larger (approximately 12000–20000), we assume that a chain shortening and breakdown occurred. This could be the result of methanolysis under the anhydrous conditions. Amino acids and xylose (from the linkage region) were present in the main product only in traces (xylose:uronic acid 1:100). Methyl glycosides formed during desulfation were subsequently split by the resin in the acidic aqueous solution. After alkaline cleavage of methyl esters and partial digestion by testicular hyaluronidase, the pattern of the products on both P-10 and P-30 columns showed poor resolution compared with corresponding fractions of oligosaccharides prepared from NaHA. Gel chromatography on P-6 of oligosaccharides prepared by a more prolonged digestion, optimized to produce mainly tetrasaccharide, showed the presence of small quantities (<20%) of additional peaks. The K_{av} values indicated the presence of oligosaccharides with an odd number of residues, which have not been apparent in the digests of HA. The appearance of these odd numbered oligosaccharides may be due to a combination of methanolysis of both hexosaminidic and glucuronidic bonds and alkaline elimination of reducing GlcNAc. This indicates that the odd oligosaccharides are derived from the ends of the chains.

Circular Dichroism Analysis. In aqueous solution at neutral pH, high molecular weight NaHA shows a single negative CD band above 200 nm, centered near 209 nm. The molar ellipticity of that band ($[\theta]_{210}$), calculated on the basis of the repeating disaccharide residue concentration, is $-10\,200 \pm 500$ deg cm^2 $dmol^{-1}$. It is essentially constant over the NaHA concentration range 0.015–9.7 mg/mL, in salt concentrations of 0.0, 0.15, and 0.40 M NaCl, and in weak phosphate buffer (pH 7.2) containing 0.145 M NaCl (Chakrabarti & Balazs, 1973; M. K. Cowman & E. A. Balazs, unpublished results). The molar ellipticity of NaHA is greatly enhanced relative to the contributions of its component monosaccharides, GlcNAc and GlcUA, whose summed ellipticity at 210 nm is -4700 ± 200 deg cm^2 $dmol^{-1}$. These data are in fairly good agreement with previously reported spectra (Chakrabarti & Balazs, 1973; Buffington et al., 1977). The CD enhancement for NaHA is not directly related to the formation of glycosides per se, since methyl α- or β-glycosides of the sugars exhibit properties nearly identical with those of the free sugars above 200 nm (Kabat et al., 1969; Morris et al., 1975; Coduti et al., 1977).

Oligosaccharides derived by enzymatic digestion of NaHA with testicular hyaluronidase exhibit CD spectra less intense than that of the polymer, with molar ellipticity values dependent on the oligosaccharide chain length. For the tetrasaccharide $(GlcUA\text{-}GlcNAc)_2$ in dilute aqueous solution, $[\theta]_{210} = -7500 \pm 300$ deg cm^2 $dmol^{-1}$. This value is unchanged over the concentration range 0.10–14 mg/mL and by increased salt concentration to 0.40 M NaCl. The CD spectrum of a tetrasaccharide prepared by a different method (Flodin et al., 1964) was also found to be equal to that of the tetrasaccharide prepared by our method. The tetrasaccharide CD spectrum is approximately equal to a calculated spectrum derived by averaging the polymer and monosaccharide contributions (Figure 3). With increasing oligosaccharide chain length ($n = 2$–8), the molar ellipticity becomes more negative (Figure 4). For $(GlcUA\text{-}GlcNAc)_8$, the largest oligosaccharide of this type examined, $[\theta]_{210} = -9100 \pm 300$ deg cm^2 $dmol^{-1}$. This value remains significantly lower than that observed for polymeric NaHA.

The chain-length dependence of the oligosaccharide CD spectra may be rationalized by consideration of the finite degrees of polymerization and resultant covalent differences of the end residues. The CD data may be analyzed according to a model which allows distinct CD contributions for the interior and end residues of the oligosaccharides. The observed molar ellipticity for each species is thus expressed as the sum of two contributions: $[\theta]_{int}$, the interior residue CD, multiplied by that fraction of the structure consisting of residues linked

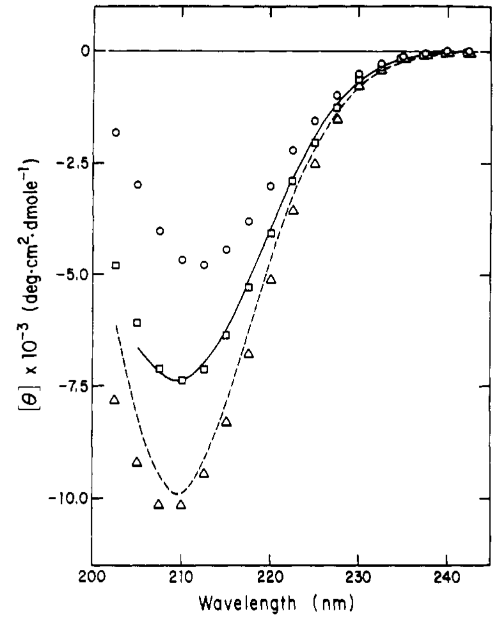

FIGURE 3: CD spectra of NaHA, monosaccharides, and tetrasaccharides in water. (—) Tetrasaccharide $(\mathrm{GlcUA\text{-}GlcNAc})_2$. (---) Tetrasaccharide $(\mathrm{GlcNAc\text{-}GlcUA})_2$. (△) NaHA. (○) Equimolar mixture of monosaccharides, GlcNAc + GlcUA. (□) Calculated average of the spectra of NaHA and GlcNAc + GlcUA. Ellipticity calculated on the basis of the uronic acid (=hexosamine) residue concentration.

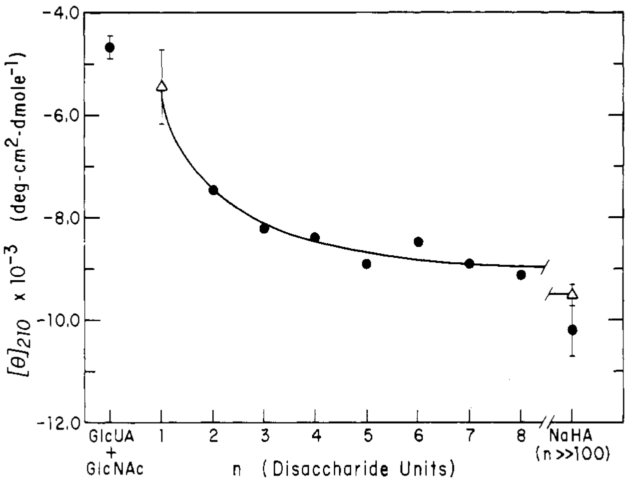

FIGURE 4: Dependence of the CD molar ellipticity at 210 nm, in water, on the degree of polymerization for oligosaccharides of the type $(\mathrm{GlcUA\text{-}GlcNAc})_n$, derived from NaHA by testicular hyaluronidase digestion. Ellipticity calculated on the basis of the uronic acid (= hexosamine) residue concentration. Standard deviation approximately ±300 deg cm² dmol⁻¹, unless otherwise indicated. (●) Experimental data. (△) Data derived by analysis of the oligosaccharide ellipticities by using a model allowing different CD contributions for interior and end sugar residues.

at both C1 and C3 or C4, and $[\theta]_{\mathrm{end}}$, the end residue CD, multiplied by the fractional occurrence of residues lacking one covalent linkage. The expression for molar ellipticity as a function of the number of repeating disaccharide residues, n, in each oligosaccharide thus becomes

$$[\theta]_{210} = [\theta]_{\mathrm{int}}(n-1)/n + [\theta]_{\mathrm{end}}(1/n)$$

or the equivalent expression

$$n[\theta]_{210} = [\theta]_{\mathrm{int}}(n-1) + [\theta]_{\mathrm{end}}$$

The value of $n[\theta]_{210}$ should thus be a linear function of $n - 1$, with a slope equal to the interior residue (disaccharide) CD contribution and an intercept equal to the end residue CD contribution. Data for the testicular hyaluronidase derived oligosaccharides showed an excellent fit to this model, with a correlation coefficient of 0.996, using a linear least-squares fitting procedure. The interior and end residue molar ellipticities were determined to be −9500 ± 200 and −5500 ± 700 deg cm² dmol⁻¹, respectively. From these values, the solid curve in Figure 4 was drawn. The end effects model thus provides an accurate description of the oligosaccharide CD data.

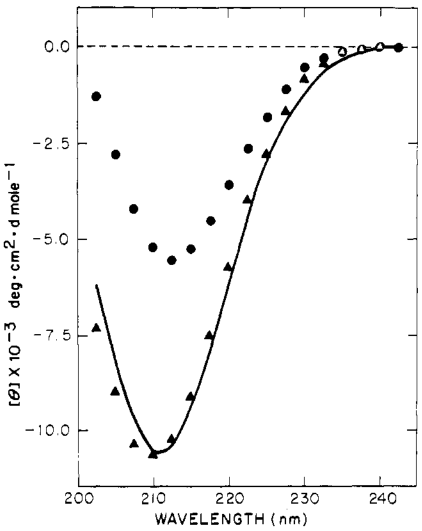

FIGURE 5: CD spectra of the trisaccharide GlcUA-GlcNAc-GlcUA and calculated equivalent spectral combinations of NaHA and monosaccharides, in water. (—) Trisaccharide GlcUA-GlcNAc-GlcUA. (▲) Equimolar mixture, the respect to uronic acid concentration, of GlcUA and NaHA. (●) Mixture of GlcNAc and two GlcUA. Ellipticity calculated on the basis of the hexosamine residue concentration, equal to one-half the uronic acid concentration.

The calculated CD properties of interior and end residues of oligosaccharides of the type $(\mathrm{GlcUA\text{-}GlcNAc})_n$ provide insight into the dependence of CD on covalent linkages between sugars. Within the experimental uncertainty, the end sugar residues are not distinguishable from free monosaccharides in solution. The best-fit value for the interior residue CD contribution is approximately 7% lower than the molar ellipticity observed for NaHA. The significance of this small difference is questionable, since it is essentially within experimental uncertainties.

The CD data for oligosaccharides produced by the action of testicular hyaluronidase may therefore be considered evidence for the following statements: (1) The structural features of high molecular weight NaHA, which result in enhanced CD intensity at 210 nm relative to its monosaccharide constituents, are shared by the interior residues of even small oligosaccharides. (2) The end sugar residues of oligosaccharides of the type $(\mathrm{GlcUA\text{-}GlcNAc})_n$ do not show a significantly enhanced CD relative to free sugars. Thus the linkages at C1 of GlcNAc and/or C4 of GlcUA are important to the CD effect in NaHA.

The CD spectrum of the trisaccharide GlcUA-GlcNAc-GlcUA is shown in Figure 5. The molar ellipticity was calculated on the basis of the disaccharide residue concentration alone, equal to one-half the uronic acid concentration. The spectrum is indistinguishable from that of a NaHA repeating disaccharide in the presence of an extra GlcUA residue. Thus, if GlcUA contributes significantly to the enhanced CD of NaHA, the residue requires only the linkage to C4 of the uronic acid. If the CD enhancement is solely related to the hexosamine moiety, as has been suggested (Buffington et al., 1977), these data serve as confirmation of the conclusion from our larger oligosaccharide data that GlcNAc linked to

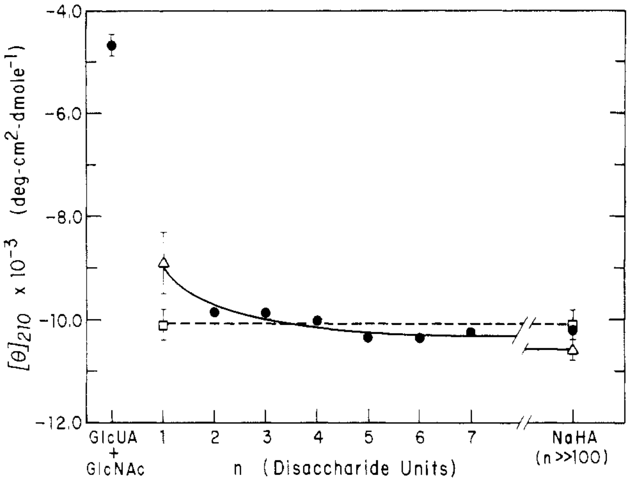

FIGURE 6: Dependence of the CD molar ellipticity at 210 nm, in water, on the degree of polymerization for oligosaccharides of the type (GlcNAc-GlcUA)$_n$, derived from NaHA by leech hyaluronidase digestion. Ellipticity calculated on the basis of the uronic acid (= hexosamine) residue concentration. Standard deviation approximately ±300 deg cm^2 dmol^{-1}, unless otherwise indicated. (●) Experimental data. (□) Extrapolated constant ellipticity equal to the mean of the larger oligosaccharide ellipticities. (△) Data derived by analysis of the oligosaccharide ellipticities by using a model allowing different CD contributions for interior and end sugar residues.

GlcUA at both C1 and C3 exhibits a CD contribution essentially equal to that of NaHA.

The importance of the linkages at C3 of GlcNAc and C1 of GlcUA to enhanced CD intensity may be determined by analysis of oligosaccharides of the type (GlcNAc-GlcUA)$_n$, produced by leech hyaluronidase digestion of NaHA. All of the oligosaccharides investigated (n = 2–7) showed a single negative CD band above 200 nm, centered near 210 nm (Figure 3), whose intensity (Figure 6) was essentially independent of chain length ($[\theta]_{210} = -10\,100 \pm 300$ deg cm^2 dmol^{-1}) and equal to that of NaHA. Thus, the interior and end residues of these oligosaccharides have approximately equal CD contributions. (If the end effects model is employed to analyze the CD data for these oligosaccharides, $[\theta]_{210}$ values of $-10\,600 \pm 200$ deg cm^2 dmol^{-1} and -8900 ± 600 deg cm^2 dmol^{-1} are obtained for the interior and end sugar residues, respectively. Within the experimental error, it is possible that the end sugars have a slightly lower molar ellipticity than the interior sugars.) The nonreducing terminal GlcNAc is linked only at C1, and the reducing terminal GlcUA is linked only at C4; thus the hexosaminidic linkage from GlcNAc to GlcUA is primarily responsible for the enhanced CD of NaHA in aqueous solution.

In a previous CD study (Chakrabarti & Balazs, 1973) of NaHA, its component monosaccharides, and the tetrasaccharide and hexasaccharide derived by testicular hyaluronidase digestion, it was speculated that the greater negative ellipticity of the polymer was indicative of a preferred conformation not found in the oligosaccharides, and therefore presumably dependent on cooperative stabilization requiring a longer chain length. The present study involving analysis of a wide series of oligosaccharides with both GlcNAc and GlcUA reducing terminals indicates that the difference between the reported CD properties of polymeric NaHA and smaller oligosaccharides of the type (GlcUA-GlcNAc)$_n$ is due to the covalent differences of the end sugars in the oligosaccharides. Specifically, the linkage from GlcNAc to GlcUA is important to the enhanced CD of NaHA. Thus, there is no evidence linking the CD properties above 200 nm to a cooperatively stabilized conformation of NaHA in aqueous solution. The normal chromatographic properties of the oligosaccharides in aqueous pyridinium acetate (Figure 1) also cast doubt on the existence of a chain-length-dependent conformation in dilute solution. If overall order in NaHA secondary structure exists, it is likely to be related to intermolecular interactions and/or the intrinsic properties of the glycosidic linkages (repetition of lowest energy angles).

In contrast to the large CD enhancement seen in NaHA as a result of the hexosaminidic linkage, the CD spectrum of the related glycosaminoglycan chondroitin is similar to that of its constituent monosaccharides. In dilute aqueous solution at neutral pH, chondroitin from either chondroitin 4-sulfate or chondroitin 6-sulfate shows a single negative CD band above 200 nm, centered near 212 nm. The molar ellipticity of that band calculated on the basis of the repeating disaccharide residue concentration is $[\theta]_{212} = -4800 \pm 200$ deg cm^2 dmol^{-1}. The summed contributions of the component monosaccharides *N*-acetyl-D-galactosamine and sodium D-glucuronate at the same wavelength equal -4100 ± 200 deg cm^2 dmol^{-1}. Thus, the chondroitin polymer shows only a 17% increase in molar ellipticity relative to the free sugars,[2] in comparison with the 117% increase observed for NaHA.

The spectroscopic difference between chondroitin and NaHA is attributed to the configurational difference in the hexosamine moiety rather than to any structural inhomogeneity resulting from the preparation (desulfation) procedure for two reasons: (1) The sulfated forms of chondroitin also show much less intense CD above 200 nm, relative to NaHA (Stone, 1971); differences between the CD of chondroitin and its sulfated forms are likely to be related to the sulfate group influence. (2) The primary products (>80%) of extensive digestion of chondroitin by testicular hyaluronidase are the expected disaccharide multiples, indicating structural regularity.

The importance of both the hexosaminidic linkage and the configuration at C4 of the hexosamine moiety to the observed CD of the nonsulfated glycosaminoglycans investigated may be explained by consideration of structural models. Across the hexuronidic linkage from GlcUA to GlcNAc, the distance of separation between the carboxylate and acetamido groups is large, whereas it is negligible across the hexosaminidic linkage. It is likely that interaction between these groups occurs in the latter case and influences the acetamido group orientation. This interaction is possible in both NaHA and chondroitin and so is not proposed to be a direct influence on the CD enhancement. The acetamido group orientation, as affected by the carboxylate group presence, may nonetheless be an indirect influence on the CD properties. Bush and co-workers (Yeh & Bush, 1974; Coduti et al., 1977; Dickinson et al., 1977) have presented evidence that the CD of free acetamido sugars is strongly affected by changes in the dihedral angle about the C-O bond at C3 of the hexosamine. In the case of nonsulfated glycosaminoglycans, this dihedral angle may be influenced by both the acetamido group orientation at C2 and the hydroxyl group configuration at C4. Because the C-O bond dihedral angle at C3 of the hexosamine is part of the glucuronidic linkage in NaHA and chondroitin, the CD differences between the two polymers may tentatively be proposed to result from differences in the average conformation about their respective glucuronidic linkages. Further

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[2] Park & Chakrabarti (1978) have reported a somewhat higher molar ellipticity ($[\theta]_{210} = -6800$ deg cm^2 dmol^{-1}) for chondroitin obtained by a different desulfation procedure (Kantor & Schubert, 1957). No comparison was made by these authors to the combined CD contributions of the monosaccharide components under the same conditions.

experimentation will be required to establish direct relationships of this type and to investigate the minimum variation in the dihedral angle resulting in altered CD properties.

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