Vacuum-Ultraviolet Circular Dichroism of Sodium Hyaluronate Oligosaccharides and Polymer Segments

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Synopsis

The CD spectrum of an enzymatically derived sodium hyaluronate (NaHA) segment preparation with chain length 18 ± 3 disaccharide units [NaHAseg, ($\xrightarrow{\beta-1,4}$ NaGlcUA $\xrightarrow{\beta-1,3}$ GlcNAc)$_{15-20}$: NaGlcUA, sodium D-glucuronate; GlcNAc, 2-acetamido-2-deoxy-D-glucose] in H_2O was recorded to 180 nm using a computer-controlled vacuum-uv CD instrument. Near 190 nm the spectrum is of low intensity, similar to the sum of the free monosaccharide contributions, attributed to the π-π^* transitions of the acetamido and carboxylate substituents. In contrast, much smaller oligosaccharides, also derived from high-molecular-weight NaHA by enzymatic digestions, show CD spectra in H_2O with prominent bands centered near 190 nm. The oligosaccharide spectra can be matched as linear combinations of interior sugar residue (= NaHAseg) and end sugar residue CD contributions. End residues from oligosaccharides of the type (NaGlcUA-GlcNAc)$_n$ show a negative CD band near 190 nm. End residues from oligosaccharides of the reverse sequence (GlcNAc-NaGlcUA)$_n$ show a positive CD band near 190 nm. Averaging of the two end-residue spectral contributions yields an approximate match for the spectrum of NAHAseg below 200 nm. It is proposed that the low intensity CD of NaHA in the π-π^* region is the result of large-magnitude, oppositely signed contributions, which can be visualized by studying oligosaccharides.

INTRODUCTION

Hyaluronic acid is a high-molecular-weight glycosaminoglycan with a linear repeating disaccharide structure, poly[(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-*O*-β-D-glucopyranuronosyl].[1] In the vitreous of the eye and the joint synovial fluid, hyaluronic acid is thought to serve as a molecular filter and a mechanical shock absorber.[2] Solutions of purified sodium hyaluronate show extremely high viscosity and unusual viscoelastic properties, indicative of specific intermolecular interactions leading to the formation of a networklike matrix.[3-6] The molecular conformation in dilute solution has been characterized as a highly hydrated

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random coil, with some stiffening influence.[7,8] On the basis of alkali-induced conformational changes,[9-11] periodate oxidation kinetics,[12] and nmr studies,[10,11,13-15] it has been tentatively proposed that the stiffening influence might involve hydrogen bonding between adjacent sugar residues.[16] Proof for hydrogen bonding and details of the chain conformation, substituent group orientations, and mechanism of interchain association remain to be established.

CD spectroscopy is a potentially useful method for the investigation of hyaluronic acid conformation in solution. The substituent acetamido and carboxylate chromophores in sodium hyaluronate (NaHA) exhibit n-π^* transitions near 210–215 nm. The CD properties of those transitions in NaHA have been rather extensively characterized by conventional methods.[17-20] The n-π^* CD appears to depend primarily on the existence of the β-1,4-glycosidic linkage, which brings the acetamido and carboxylate groups into close proximity.[20] These conclusions are subjected to reinvestigation in the present study, insofar as band overlap from higher-energy transitions had not been fully analyzed.

The acetamido and carboxylate π-π^* absorption transitions occur near 180–190 nm. It has previously been reported[21] that the NaHA polymer spectrum is of low intensity in the π-π^* transition region. Near 190 nm, the spectrum of NaHA appears reminiscent of the summed contributions of the two component free monosaccharides. This observation is surprising, in view of the change in n-π^* CD and the documented sensitivity of the hexosamine acetamido chromophore π-π^* CD to the presence of glycosidically linked sugar residues in other complex carbohydrate structures.[22-24] In addition, Scott and Rees and their coworkers[10-16] have presented evidence suggestive of a hydrogen-bonding pattern in NaHA that involves the acetamido and carboxylate chromophores, thereby forcing an alteration in orientation relative to their respective sugar rings. We have considered that the absence of any notable CD difference near 190 nm between the polymer and its monosaccharide components might be coincidental, resulting from a cancellation of underlying opposite-sign spectral contributions. In the present study, the 180–240-nm CD spectra of oligosaccharide fragments of NaHA have been analyzed as a function of chain length and sequence. The spectra were obtained using a vacuum-uv CD instrument optimized for data acquisition in that wavelength range.[25,26] Evidence is presented that the polymer spectrum may contain masked opposing CD contributions, related to the influence of the formation of the two distinct glycosidic linkages on the optical properties of the acetamido and/or carboxylate groups in the repeating structure.

MATERIALS AND METHODS

Monosaccharides

2-Acetamido-2-deoxy-D-glucose (GlcNAc) and sodium D-glucuronate (NaGlcUA) were obtained from Sigma Chemical Company.

Sodium Hyaluronate

High-molecular-weight sodium hyaluronate (NaHA) was obtained from Pharmacia (Piscataway, N.J.). This sample (lot number DL 10492) was described previously.[20] Prior to CD analysis, an aliquot of NaHA (at 9.7 mg/mL concentration) was dialyzed overnight at 4°C against several changes of distilled water.

Preparation of Sodium Hyaluronate Segment (NaHAseg)

The hyaluronate segment (enzymatically derived NaHA segment preparation with chain length 18 ± 3 disaccharide units) was obtained by enzymatic digestion of high-molecular-weight NaHA that had been prepared from rooster combs by the method of Balazs.[27,28] The procedure for NaHA digestion by bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, E.C. 3.2.1.35) was essentially that described[20] for an earlier preparation of smaller oligosaccharides, except that only two additions of enzyme at $t = 0$ and $t = 1$ h were made, with a total digestion time of 2 h.

NaHAseg was prepared from the digestion mixture by chromatography on a 2.5 × 195-cm column of Bio-Gel P-60 (100–200 mesh) in 0.5*M* NaCl, at a flow rate of 20 mL/h. Fractions of 4 mL were collected and screened for uronic acid content. This column fractionates hyaluronate oligosaccharides ranging in size from the tetrasaccharide ($K_{av} = 0.75$) to segments containing approximately 20 disaccharide units ($K_{av} \simeq 0.03$). Discrete oligosaccharide peaks are observable for species up to 14 disaccharides in length ($K_{av} = 0.21$). For our hyaluronate segment, fractions from $K_{av} = 0.03$ to 0.20 were pooled, corresponding in size to approximately 14–20 disaccharide units in length. This material represented approximately 40% of the total digest products. The pooled segment fractions were concentrated by rotary evaporation and loaded on a 1.5 × 200-cm column of Bio-Gel P-2 (100–200 mesh) in water for desalting. Fractions of 3.2 mL were collected at a flow rate of 20 mL/h and screened for uronic acid content. The desalted segments were lyophilized and stored at room temperature *in vacuo* over P_2O_5. The average chain length of NaHAseg was determined to be 18 disaccharide units by analysis of the molar ratio of uronic acid residues to reducing terminal N-acetyl hexosamine.

Preparation of $(GlcNAc\text{-}GlcUA)_n$ Oligosaccharides

NaHA was digested by purified leech head hyaluronidase (hyaluronate 3-glycanohydrolase, E.C. 3.2.1.36) as described elsewhere.[20] The chromatographic separation of the oligosaccharides employed a 1.5 × 195-cm column of Bio-Gel P-6 (200–400 mesh) equilibrated in 0.5*M* pyridinium acetate, pH 6.5, at a flow rate of 8 mL/h. Approximately 25 mg of the digest products was applied to the column. Fractions of 2.5 mL were collected

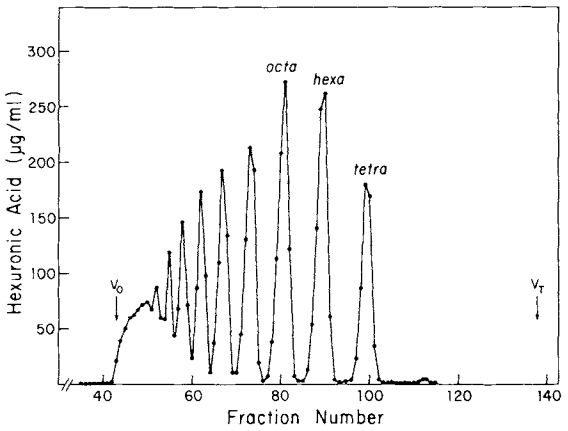

Fig. 1. Separation of oligosaccharides produced by digestion of NaHA with leech hyaluronidase. Approximately 25 mg of sample was applied to a column (1.5 × 195 cm) of Bio-Gel P-6, 200–400 mesh, and eluted with 0.5*M* pyridinium acetate, pH 6.5. The peaks are identified according to the species chain length, determined by chemical analysis.

and screened for uronic acid content. Baseline separation of oligosaccharides containing 2–6 disaccharide units was achieved (Fig. 1). Appropriate fractions were pooled, dried, and water-washed by rotary evaporation, converted to the Na^+ salt on Bio-Rad AG 50WX8 resin, lyophilized, and stored over P_2O_5 *in vacuo* at room temperature. Chain lengths of the oligosaccharides were determined by analysis of the fraction of total uronic acid lost on $NaBH_4$ reduction of the terminal residue. For the tetrasaccharide, hexasaccharide, and octasaccharide fragments, reduction resulted in a loss of 48% (theory, 50%), 32% (theory, 33%), and 25% (theory, 25%), respectively, of the total uronic acid.

Preparation of (GlcUA-GlcNAc)$_n$ Oligosaccharides

Exhaustive digestion of NaHA by testicular hyaluronidase proceeded for a total of 72 h, with enzyme additions at $t = 0$, 5, and 48 h, using essentially the method described.[20] The digest was chromatographed on Bio-Gel P-6 as above and yielded only the well-separated tetrasaccharide and hexasaccharide species. The molar ratios of uronic acid residues to reducing terminal *N*-acetyl hexosamine for these samples were 1.8 and 2.7, respectively.

Chemical Analysis

Procedures for chain-length determination, purity analyses, and concentration determination were previously described.[20]

Spectroscopic Analysis

CD spectra were measured at room temperature for water solutions of samples in quartz sandwich cells of 10-μm nominal pathlength, using a vacuum-uv instrument that has a nitrogen flushed sample chamber.[25] The instrument was calibrated at 192.5 and 290.5 nm with d-10 camphor sulfonic acid.[29] The CD spectrum for each sample was automatically signal-averaged from 4–8 repetitive scans[26] and smoothed by a Fourier digital filtering procedure.[30] Before smoothing, the signal-to-rms noise ratios were approximately 30:1 at 210 nm and 10:1 at 180–190 nm, using a time constant of 3 s. Spectra described in this manuscript are the means of the smoothed and scaled data for two or three identically prepared samples.

CD scaling factors containing a composite concentration and pathlength term were obtained from absorption data measured for each sample using the CD spectrophotometer[26] immediately prior to CD analysis. Extinction coefficients were determined using a Cary model 15 spectrophotometer under nitrogen purge. A value of $\epsilon_{188} = 1.1 \times 10^4$ L cm^{-1} mol^{-1} was found applicable for all oligosaccharide samples and NaHAseg. As a test of the accuracy of the CD scaling factors so determined, the concentration of each sample was measured by uronic acid analysis, and the optical cell pathlength calculated from the absorption data. The average pathlength was found to be 10.4 μm, with a standard deviation of 1.7 μm. This is in reasonable agreement with the nominal cell pathlength. Additional measurements using a sandwich cell of 100-μm nominal pathlength yielded a calculated length of 89 μm. Spectra obtained for separately prepared repeat samples deviated by no more than 10% from the mean value at the 210-nm minimum.

For GlcNAc, the CD scaling factor was also determined from its absorption spectrum, using $\epsilon_{188} = 8.0 \times 10^3$ L cm^{-1} mol^{-1}. For NaGlcUA, the absence of a clear shoulder above 180 nm in the absorption spectrum precluded accurate determination of a CD scaling factor by this method. In this case, the nominal cell pathlength of 10 μm was assumed, and the dry weight concentration employed. The ellipticity calculated for NaGlcUA at 215 nm is approximately 40% greater than our previously reported value.[20] A similar discrepancy was noted with all samples in this study, and it appears to reflect a difference in calibration of the Cary 60 previously employed. No alterations in our earlier conclusions based on relative sample ellipticities are required on this basis.

RESULTS

NaHAseg and Monosaccharide CD Spectra

The 180–240-nm CD spectrum of NaHAseg in aqueous solution at neutral pH is shown in Fig. 2. Within experimental error, the spectrum was found to be identical for sample concentrations of 3 and 30 mg/mL. A

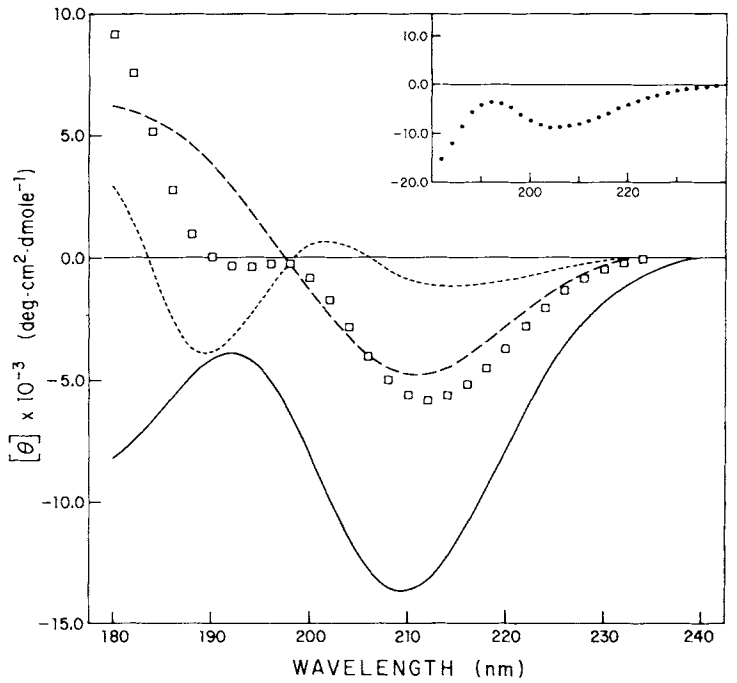

Fig. 2. Comparison of CD spectra of NaHA segments and monosaccharides: (—) NaHAseg, (– –) GlcNAc, (- - -) NaGlcUA. (□) Calculated sum of GlcNAc and NaGlcUA spectra. Inset: Calculated difference spectrum, NaHAseg − (GlcNAc + NaGlcUA).

single negative band is observed above 200 nm, centered near 209 nm, in agreement with previous reports for NaHA.[17-21] A point of minimal ellipticity occurs near 192 nm, and a portion of a second negative band centered below 180 nm is apparent. The NaHAseg spectrum presented here differs with respect to band shape in the 180–190-nm region from a previously reported[21] vacuum-uv CD spectrum for high-molecular-weight NaHA. A new direct comparison of polymer and polymer segment spectra, both studied under the same conditions, failed to show any significant difference between the two species.

The CD spectra obtained for 2-acetamido-2-deoxy-D-glucose (GlcNAc) and sodium D-glucuronate (NaGlcUA) are also shown in Fig. 2, for comparison with the NaHAseg spectrum. The spectrum of GlcNAc is in good agreement with earlier observations.[22] It exhibits a negative band near 212 nm, due to the acetamido n-π^* transition. Crossover occurs near 198 nm, followed by a peak centered at or below 180 nm. This latter band contains a contribution from the acetamido π-π^* transition (absorption shoulder at approximately 188 nm), as well as some overlap below 190 nm from chromophore transitions common to simple hexoses.[31-33] The spectrum of NaGlcUA contains at least four CD bands, alternately positive and negative. The two lowest energy bands have been attributed to the

n-π^* transition for two different rotational isomers of the carboxylate group.[34-37] In the shorter wavelength region, the contributions include the carboxylate π-π^* band(s) and the hexose transitions. The severe overlap of bands makes a precise analysis of the carboxylate CD properties impossible, but the band apparently centered near 190 nm presumably derives from the π-π^* transition. No clear absorption maximum was visible to 180 nm, as a result of strong background absorption from the pyranose-ring chromophoric groups, so that exact location of the π-π^* band could not be made.

The sum of the free monosaccharide spectra is a poor match for the spectrum of NaHAseg (Fig. 2). The overall spectral perturbation of sugar residues in NaHAseg relative to the sum of the free sugar components can be seen from the calculated difference spectrum. The pattern of change appears simple: the addition of negative ellipticity (or equivalently, reduction of positive ellipticity) centered near 205 nm, and near or below 180 nm. The change above 200 nm has been investigated previously.[20] It was found to depend on the existence of the β-1,4-glycosidic linkage from GlcNAc to NaGlcUA and any resultant conformational changes. For the spectral perturbation below 190 nm, the change from the hemiacetal chromophore of the free monosaccharide to the acetal chromophore of the glycoside must be considered. Johnson[33,38] has previously shown that formation of β-methyl glycosides of D-hexoses ($^4C_1$ chair) with equatorial substituents at C2 results in a negative CD perturbation below 190 nm, albeit less intense than that observed here. On the basis of the calculated difference spectrum between NaHAseg and the free monosaccharides, there are no apparent alterations in the acetamido or carboxylate π-π^* CD properties, which would be anticipated to lie near 190 nm, as previously noted by Buffington et al.[21] The 190-nm region has been further investigated in oligosaccharides of NaHA.

Oligosaccharide CD Spectra

The spectra obtained for the tetrasaccharide $(NaGlcUA\text{-}GlcNAc)_2$ and hexasaccharide $(NaGlcUA\text{-}GlcNAc)_3$ derived by digestion of NaHA with testicular hyaluronidase are shown in Fig. 3. A clear negative band in the π-π^* region is observed for these species. In the tetrasaccharide case, the negative band is centered at 187–188 nm, with a molar ellipticity of $[\theta]_{187.5} = -1.44 \times 10^4$ deg cm^2 dmol^{-1}, calculated on the basis of the repeating disaccharide residue concentration. The hexasaccharide also exhibits a negative band at 187 nm, with $[\theta]_{187} = -1.01 \times 10^4$ deg cm^2 dmol^{-1}.

Spectra obtained for oligosaccharides of reversed sequence, derived by enzymatic digestion of NaHA with leech hyaluronidase, show that there is a strong sequence dependence to the π-π^* region CD, in addition to a chain-length dependence (Fig. 4). The tetrasaccharide $(GlcNAc\text{-}NaGlcUA)_2$ has a positive CD band at 192–193 nm, with a molar ellipticity of $[\theta]_{192.5} = +5.3 \times 10^3$ deg cm^2 dmol^{-1}. The hexasaccharide (GlcNAc-

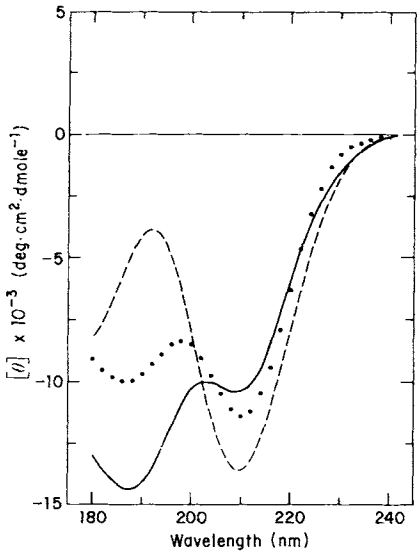

Fig. 3. CD spectra of NaHAseg and oligosaccharides of the type $(NaGlcUA - GlcNAc)_n$, derived from NaHA by testicular hyaluronidase digestion: (– –) NaHAseg, (—) tetrasaccharide $(GlcUA\text{-}GlcNAc)_2$, (· · ·) hexasaccharide $(GlcUA\text{-}GlcNAc)_3$.

$NaGlcUA)_3$ and the octasaccharide $(GlcNAc\text{-}NaGlcUA)_4$ exhibit progressively less intense positive CD bands in that region, with molar ellipticities of $[\theta]_{192.5} = +1.5 \times 10^3$ deg cm^2 dmol^{-1} and $[\theta]_{192} = +0.3 \times 10^3$ deg cm^2 dmol^{-1}, respectively.

Calculated End-Effects in Oligosaccharide Spectra

For each oligosaccharide sequence type, the observed CD spectrum in the π-π^* region tends toward that of the polymer segment with increasing oligosaccharide chain length. That trend suggests the possibility that for each chain-length species, the observed spectrum is the weighted summation of two distinct components: (1) a contribution from the interior sugar residues and (2) a sequence-dependent contribution from the end-sugar residues. Thus, the molar ellipticity at a given wavelength for any oligosaccharide would be expressed as

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

where n is the number of repeating disaccharide units and $[\theta]_{end}$ is sequence dependent. In principle, the applicability of the "end-effect" model and the values of the interior and end-residue CD contributions can be determined from a plot of $n[\theta]$ versus $n - 1$ for a given sequence. An earlier analysis[20] of the n-π^* CD of NaHA oligosaccharides through $n = 8$ by this procedure showed that this model works well at 210 nm. At that wave-

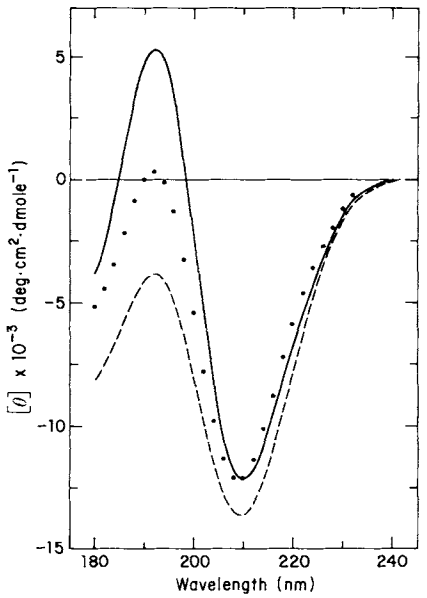

Fig. 4. CD spectra of NaHAseg and oligosaccharides of the type $(GlcNAc - NaGlcUA)_n$, derived from NaHA by leech hyaluronidase digestion: (– –) NaHAseg, (—) tetrasaccharide $(GlcNAc\text{-}NaGlcUA)_2$, (· · ·) octasaccharide $(GlcNAc\text{-}NaGlcUA)_4$.

length, the interior residue contribution is indistinguishable from that of NaHA, and the end-residue contribution is a function of the oligosaccharide sequence.

The present data set is too small to allow accurate analysis of the π-π^* region by this data-fitting procedure. The end-sugar CD contributions can, however, be estimated by assuming the interior-sugar CD contribution to be equal to that of NaHA above 180 nm. That is, the interior-sugar residues are assumed to be conformationally equivalent to polymer segment residues, with respect to the immediate environments of the chromophores. The end-sugar contributions may then be calculated from the polymer segment and oligosaccharide spectra, i.e.,

$$[\theta]_{\text{end}} = n[\theta] - (n - 1)[\theta]_{\text{NaHAseg}}$$

The CD contributions calculated for the two sequence types of end residues, determined from the corresponding tetrasaccharide spectra and the polymer segment spectrum, are shown in Fig. 5.

The lower energy CD band seen in the calculated end-residue spectra is attributed to the n-π^* transitions of the acetamido and carboxylate groups. To approximately 215–220 nm, its apparent intensity is affected by overlap from the higher energy bands. In the end-residue sequence NaGlcUA $\xrightarrow{\beta\text{–}1,\ 3}$ GlcNAc, the resolved n-π^* band would be slightly less intense than the observed value of $[\theta]_{210} = -7.2 \times 10^3$ deg cm^2 dmol^{-1}, with

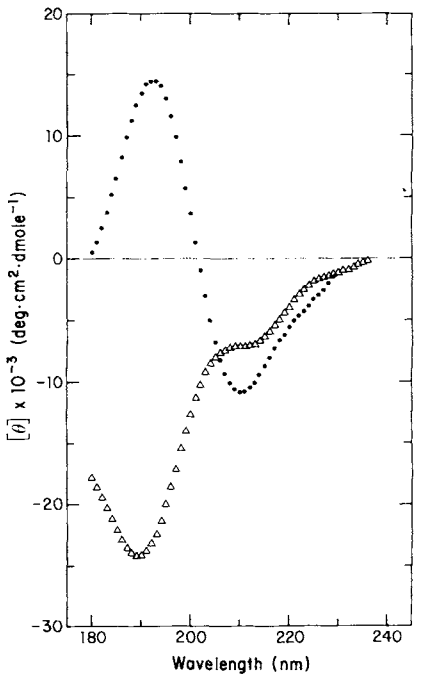

Fig. 5. Oligosaccharide end-residue CD contributions, calculated from the spectra of NaHAseg and the corresponding tetrasaccharide: (△) NaGlcUA $\xrightarrow{\beta\text{-}1,}$ $\xrightarrow{3}$GlcNAc, (●) GlcNAc $\xrightarrow{\beta\text{-}1,}$ $\xrightarrow{4}$NaGlcUA.

a molar ellipticity closer to the sum of the free monosaccharide (GlcNAc + NaGlcUA) contributions ($[\theta]_{210} = -5.6 \times 10^3$ deg cm^2 dmol^{-1}) in that wavelength region. For the reversed residue sequence GlcNAc $\xrightarrow{\beta\text{-}1,}$ $\xrightarrow{4}$ NaGlcUA, the resolved n-π^* band would be slightly more intense than the observed value of $[\theta]_{210} = -1.1 \times 10^4$ deg cm^2 dmol^{-1}, with a molar ellipticity nearly equal to that of polymeric NaHA residues ($[\theta]_{210} = -1.4 \times 10^4$ deg cm^2 dmol^{-1}). These observations confirm the results of an earlier investigation[20] into the structural dependence of the n-π^* CD in NaHA and its oligosaccharides, which showed that the GlcNAc $\xrightarrow{\beta\text{-}1,4}$ NaGlcUA linkage is important for the determination of the polymer residue CD intensity at 210 nm.

The higher-energy CD bands observed in the calculated end-residue spectra of Fig. 5 are primarily attributed to the acetamido and/or carboxylate π-π^* transitions. For the end-residue sequence NaGlcUA $\xrightarrow{\beta\text{-}1,}$ $\xrightarrow{3}$ GlcNAc, a negative band is located at 190 nm, with a molar ellipticity of $[\theta]_{190} = -2.4 \times 10^4$ deg cm^2 dmol^{-1}. The reversed sequence GlcNAc $\xrightarrow{\beta\text{-}1,}$ $\xrightarrow{4}$ NaGlcUA exhibits a positive CD band with a peak at 192 nm, having $[\theta]_{192} = +1.4 \times 10^4$ deg cm^2 dmol^{-1}. Neither of these large-magnitude bands is observed in the NaHA polymer segment.

TABLE I
Calculated Oligosaccharide End-Residue π-π^* CD[a]

End-Residue	Oligosaccharide	λ_{max} (nm)	$[\theta]$ (deg cm^2 dmol^{-1})
NaGlcUA $\xrightarrow{\beta\text{-}1,}$ $\xrightarrow{3}$ GlcNac	(NaGlcUA-GlcNAc)$_2$	189.5	-2.42×10^4
	(NaGlcUA-GlcNAc)$_3$	190.0	-2.10×10^4
GlcNAc $\xrightarrow{\beta\text{-}1,}$ $\xrightarrow{4}$ NaGlcUA	(GlcNAc-NaGlcUA)$_2$	192.5	$+1.45 \times 10^4$
	(GlcNAc-NaGlcUA)$_3$	194.0	$+1.27 \times 10^4$
	(GlcNAc-NaGlcUA)$_4$	192.0	$+1.27 \times 10^4$

[a] End-residue CD calculated assuming interior-residue CD equal to the CD of NaHA; $[\theta]_{end} = n[\theta]_{oligo} - (n-1)[\theta]_{NaHAseg}$, where n is the number of repeating disaccharide units in the oligosaccharide structure.

As a test of the "end-effect" analysis of the tetrasaccharide CD spectra, the larger oligosaccharides were similarly analyzed. The band shape, position, and intensity of the end-residue contributions calculated from the hexasaccharide and octasaccharide spectra were found to be in good agreement with the data obtained from the tetrasaccharide spectra, despite the greater error involved in analyzing end effects for larger fragments (Table I).

DISCUSSION

Oligosaccharide Interior Residue CD

The CD spectra of NaHA oligosaccharides studied in neutral aqueous solution can be satisfactorily analyzed on the basis of an "end-effect" model. In this analysis, the CD contribution due to the oligosaccharide interior-sugar residues is assumed to be equal to that of NaHA polymer segment residues. On the basis of the success of the end-effect model, there is no discernible contribution to the polymer segment CD, which reflects a cooperatively stabilized ordered conformation. Furthermore, in a limited study we observed no significant difference between the CD spectra of NaHAseg ($MW \sim 7000$) and high-molecular-weight NaHA ($MW \sim 2.4 \times 10^6$) examined under the same conditions. The segment preparation was chosen for direct comparison with the oligosaccharide samples, since the possibility of variable contributions to the polymer CD as a function of chain–chain interactions or conformational aspects unique to the very high molecular weight material lies outside the scope of the present investigation.

Oligosaccharide End-Residue CD

The calculated end-residue contributions to the CD spectra of NaHA oligosaccharides contain strong CD bands centered near 190 nm. These

bands are primarily attributed to the π-π^* transitions of the acetamido and/or carboxylate chromophores. The optical activity of those transitions differs in the oligosaccharide end residues, in comparison with the free monosaccharides. There are several possible sources of the alteration in π-π^* CD, including (1) change in average orientation of the chromophoric group(s) with respect to the hexopyranose ring(s), (2) conformational change(s) in the hexopyranose ring(s), and (3) new interchromophoric interactions, including possible coupling between the acetamido and carboxylate groups, as well as coupling of those chromophores with the newly formed acetal group. Conformational changes involving the hexose ring and/or its substituents have previously been proposed by Buffington et al.[21] to explain the n-π^* transition CD of hyaluronate.

There are currently no data available in the literature that directly establish the average orientations of the acetamido or carboxylate groups in NaHA in aqueous solution. Hydrogen bonding between those chromophores, as suggested by Scott and Rees and their coworkers,[10-16] may change the preferred orientations. For the carboxylate group, the equilibrium between the two proposed[34-37] conformers would presumably be altered, or a new orientation adopted. Hydrogen bonding would also result in a significant deviation[16] in the acetamido group orientation from that observed in monomeric GlcNAc (amide proton approximately *trans* to the ring proton).[39-42] Linkage-dependent changes in the acetamido group orientation that may be unrelated to hydrogen-bond formation must also be considered. Conformational calculations show a broad minimum of energy for the *trans* ($\tau_2 = 120°$) conformation and a narrow minimum for the conformation in which the two protons are *cis*.[43] The conformational freedom within the *trans* region, for which the dihedral angle τ_2 can vary from 100° to 150°, is sufficiently great to allow substantial changes in the CD.

With regard to the hexopyranose ring conformations, Welti et al.[10] have determined ring proton–proton coupling constants in D_2O after computer simulation of the 300-MHz nmr spectrum. Those authors judged the GlcNAc and GlcUA ring conformations to be similar in NaHA and methyl glycosides of the monosaccharides. The conformations of β-methyl GlcNAc and α- or β-GlcNAc are also similar in D_2O.[44] However, small deviations in ring conformation could conceivably result in significant CD changes.

With regard to the formation of new interchromophoric interactions, the change from the free monosaccharide hemiacetal group to the glycosidic acetal group involving C1 could theoretically alter the CD of either the acetamido or carboxylate chromophore on the same sugar. Methyl glycosides of the monosaccharides serve as appropriate models for this chromophoric change. The β-methyl glycoside of GlcNAc studied in aqueous solution[22] shows a CD spectrum that is very similar in shape to the calculated spectrum for the GlcNAc $\xrightarrow{\beta\text{-}1,\ 4}$ NaGlcUA end residues, but approximately one-third as intense. The noted similarity in spectral

shape may indicate the importance of the new interchromophoric (C1) acetal–acetamido interaction to the observed CD. For the NaGlcUA $\xrightarrow{\beta\text{-}1,\ 3}$ GlcNAc structure, the β-methyl glycoside of NaGlcUA is a relevant model. This glycoside has not yet been examined by vacuum-uv CD, but conventional CD measurements[37] to 190 nm are very similar to our free monosaccharide spectrum. An alternative source of a new interchromophoric interaction for this end-residue structure is coupling of the acetal linkage at C3 of the GlcNAc residue with the acetamido group at C2. A simple structure that might provide a useful comparison in this case is Gal $\xrightarrow{\beta\text{-}1,3}$ GalNAc. The CD spectrum of that compound[24] shows an intense negative band at 180 nm.

In general, it should be considered that differences between the spectra of the end residues and the free monosaccharides reflect new interchromophoric interactions, as well as possible alterations in existing interactions as a result of conformational changes accompanying formation of the glycosidic linkages.

Relationship Between Oligosaccharide End-Residue and NaHA Polymer Segment CD

The large-magnitude π-π^* CD bands observed near 190 nm in oligosaccharide end residues are not visibly retained in the polymer segment spectrum. The difference spectrum for NaHAseg relative to unlinked monosaccharides shows little apparent spectral change at that wavelength. It therefore becomes of interest to consider the fate of the spectral perturbations seen in the oligosaccharide end residues, when those residues become fully linked into a polymer structure.

The NaHAseg spectrum is intermediate in shape and intensity, in the π-π^* and hexose transition regions, relative to the two opposite-sequence tetrasaccharides. Below 200 nm, an average of the two tetrasaccharide spectra provides an approximate match for the NaHAseg spectrum (Fig. 6). Similarly, an average of the two corresponding hexasaccharide or opposite sequence end-residue spectra nearly matches that of NaHA in that region. It is difficult to interpret such a relationship at present, since the structural dependence of the π-π^* CD for acetamido sugars or uronic acids has not been quantitatively treated by theory. It is possible, however, to speculate that the optical activities of the chromophoric groups of NaHA are perturbed on glycosidic linkage formation, in a manner similar to that observed in the oligosaccharide end residues. The perturbations related to formation of the two distinct linkages could fortuitously cancel to yield the low intensity polymer spectrum. Thus, the polymer spectrum may effectively contain masked CD contributions, the general nature of which can be visualized by analysis of oligosaccharides. These masked contributions are *linkage dependent* and therefore supplant the simple cancellation of opposite-sign CD contributions due to the free monosaccharides

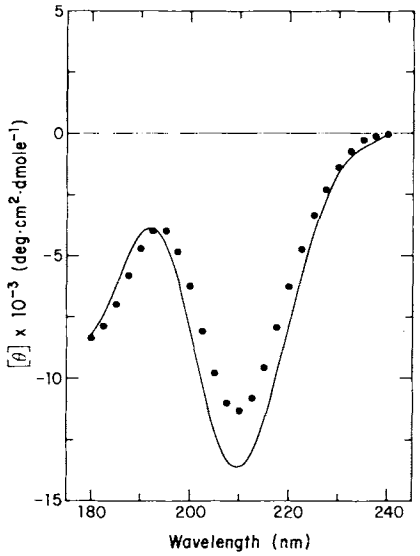

Fig. 6. Comparison of the CD spectrum of NaHAseg with the average of the two tetrasaccharide spectra: (—) NaHAseg, (●) calculated average of the tetrasaccharides = 0.5 [(NaGlcUA-GlcNAc)$_2$ + (GlcNAc-NaGlcUA)$_2$].

GlcNAc and NaGlcUA, proposed by Buffington et al.[21] to result in the low-intensity CD of NaHA near 190 nm.

Glycosaminoglycan CD

Stipanovic and Stevens[45] recently reported the vacuum-uv CD spectrum of chondroitin, the 2-acetamido-2-deoxy-D-galactose analog of NaHA. We have confirmed their observations, and found that the chondroitin and NaHAseg spectra are nearly superimposable in the π-π^* region. The NaHAseg spectrum, however, is much more intense in the n-π^* region, as previously reported by Cowman et al.[20] It is reasonable to propose that the structurally similar chondroitin may also have masked linkage-dependent perturbations of the π-π^* transitions. The masked contributions in chondroitin need not be identical to those found in NaHA, but need only be mutually offsetting.

Other glycosaminoglycans may have hidden CD contributions in the π-π^* transition region. In an early comparative study of glycosaminoglycan CD in neutral aqueous solution, Stone[17] found evidence of optical activity near 190 nm, the sign of which could be correlated with the positions and configuration (α or β) of the linkages to the acetamido sugar. Glycosaminoglycans in which the acetamido sugar is linked at C1 (β) and C3 to GlcUA (hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate) show weak negative CD below 200 nm, in agreement with our observed NaHA spectrum. Dermatan sulfate, in which the predominant repeating unit contains GalNAc linked at C1 (β) and C3 to IdUA, shows a more intense negative

ellipticity below 200 nm. In contrast, glycosaminoglycans in which the acetamido sugar is linked at C1 (α or β) and C4 (heparin, heparan sulfate, keratan sulfate) show positive ellipticity near 190 nm.

Because the π-π^* CD for glycosaminoglycans is well correlated with the acetamido sugar linkage pattern, that chromophoric group may be the primary determinant of the 190-nm CD. The CD spectra of other complex carbohydrates containing acetamido sugars in the absence of uronic acid residues show a similar dependence of the 190-nm CD on the substitution pattern, when studied in aqueous solution. Thus, GlcNAc oligomers in which the residues are linked at C1 (β) and C4 show a positive CD band near 190 nm[22]; GalNAc residues linked to a neutral sugar at C3 exhibit negative ellipticity in the π-π^* region[24]; GlcNAc residues linked to neutral sugars at both C1 (β) and C3 exhibit low-magnitude (positive) ellipticity near 190 nm.[24] The coupling of the acetamido chromophore with the acetal chromophores of the glycosidic linkages may be of paramount importance to the CD spectra of those compounds, as well as the glycosaminoglycans. The observed spectra should therefore be a function of the relative orientations of those groups.

The π-π^* CD of glycosaminoglycans can be altered in the solid state or in other solvent systems.[21,45] In general, the spectra vary between the extremes observed in our two opposite-sequence oligosaccharide end-residues. Changes in polymer conformation in response to changes in solvent environment may result in altered interchromophoric interactions, leading to the enhancement or diminution of one or both of the CD contributions, which fortuitously offset one another in aqueous solution. A more detailed theoretical and experimental analysis of the covalent and conformational features reflected in oligosaccharide end-residue CD spectra may clarify the nature of conformational changes observed in the polymer by allowing a separation of the effects associated with each of the two distinct glycosidic linkages.

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