reaction as the source of the hydride impurity. Thus, we conclude that the hydride impurity originates in reactions involving water and probably occurs during the slow (48 h) $(SN)_x$ crystal growth–solid state polymerization process used in the synthesis of $(SN)_x$ crystals.[3a] This is supported by our failure to observe a hydride impurity from amorphous S_2N_2 at 273 K.

In addition to the hydride impurities, at least two other impurities are observed. Ions at *m/e* 62 and 63 (possibly $SNOH^+$) in the "direct method" and "indirect method" spectra are observed. These ions are much more intense in the "indirect method" spectra and the ion currents decrease with the time the sample is heated under vacuum. Another species at *m/e* 140 (possibly $N_2S_3O^+$) was observed in the "indirect method" spectra but decreased at a different rate than *m/e* 63 when $(SN)_x$ was heated under vacuum. Both impurities were not detectable after 10–15 h under vacuum at 140 °C. These facts suggest that the *m/e* 140 impurity originates from a reaction involving the glass surfaces available in the "indirect method". We conclude that, in contrast to the hydride impurity, these impurities are not inherent to the $(SN)_x$ crystals.

In summary, the present work provides evidence for a new gaseous $(SN)_4$ species, of possibly "linear" bent-chain structure, as the predominant component of $(SN)_x$ vapor. The heat of sublimation of $(SN)_x$ has been determined to be 29.0 ± 0.5 kcal/mol and the gas-phase chemistry of $(SN)_x$ vapor has been discussed. Evidence has been presented for the existence of other species, including at least two impurities in the $(SN)_x$ crystals.

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Far-Ultraviolet Circular Dichroism of *N*-Acetylglucosamine, Glucuronic Acid, and Hyaluronic Acid

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Abstract: Circular dichroism spectra are reported for *N*-acetylglucosamine in solution (above 175 nm), glucuronic acid in solution (above 185 nm) and in films (above 175 nm), and hyaluronic acid in solution (above 180 nm) and in films (above 150 nm). The observed features are assigned to n–π* and π–π* transitions of the amide and carboxyl chromophores. There is a nonadditivity in the optical features of the polysaccharide relative to the monomer spectra, and possible sources of this nonadditivity are discussed. The amide π–π* transition has large negative rotational strength in hyaluronate films, the origin of which is attributed to a decreased rotational freedom of the acetamido group when it participates in an intramolecular hydrogen bond in the solid state helical structure. That transition is only weakly optically active in solution indicating that the acetamido groups are not involved in stabilizing the stiff domains of the polysaccharide chain.

Hyaluronic acid is a linear polysaccharide of the form $(G-N)_n$ where G is glucuronic acid (D-glucopyranosyluronic acid) and N is *N*-acetylglucosamine (2-acetamido-2-deoxy-D-glucopyranose). The G–N linkage is $\beta(1 \rightarrow 3)$ and the N–G linkage is $\beta(1 \rightarrow 4)$. The polysaccharide is found in the intercellular matrix of the connective tissues of most vertebrates and in some bacterial capsules.

X-ray studies[2] indicate the presence of helical structure in stretched films of hyaluronates. The detailed structure in solution is not known, but the rheological behavior of solutions is rather unusual and depends on pH as well as concentration.[3,4] Helical structure probably does not persist in solution[5] and a recent NMR study[6] indicates that in solution the chain can be described as having two domains, one of which is flexible and the other stiff. The structure of the stiff domain is as yet uncharacterized.

A previous CD[7] study[8] indicated that the magnitude of the ellipticity at 210 nm, per disaccharide, is larger in hyaluronic acid than in its tetramer and hexamer degradation products. Whether this nonadditivity in the optical property has any relation to the presence of stiff domains in the chain, or reflects purely localized perturbations in the ring conformation or the

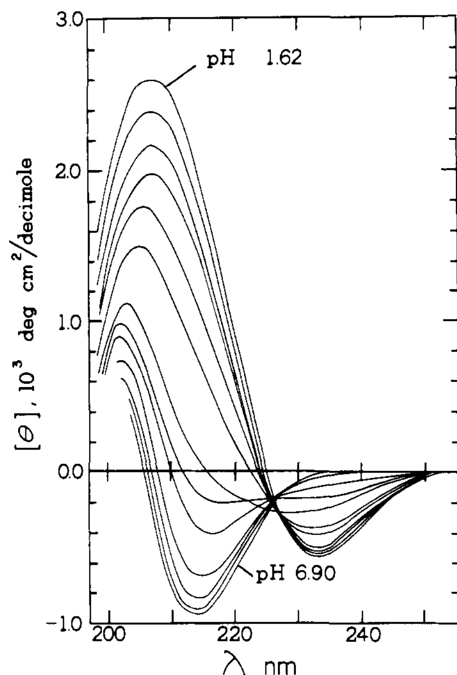

Figure 1. Circular dichroism titration for glucuronic acid. Spectra are reported for the following pH values: 1.62, 2.04, 2.50, 2.78, 3.02, 3.14, 3.48, 3.52, 3.75, 4.22, 4.60, 6.00, 6.90.

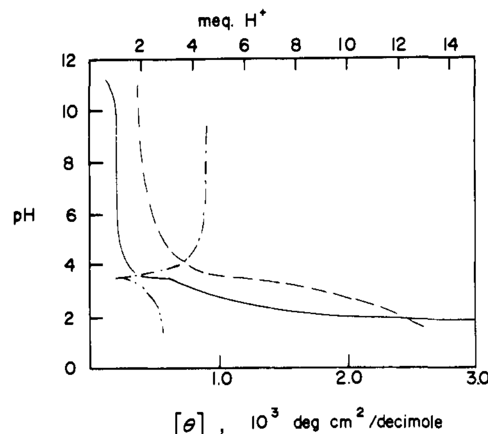

Figure 2. Titration curve for glucuronic acid (—). pH dependence of the circular dichroism positive extremum (- - -). pH dependence of the circular dichroism negative extrema near 214 nm (-··-) and 233 nm (-···-).

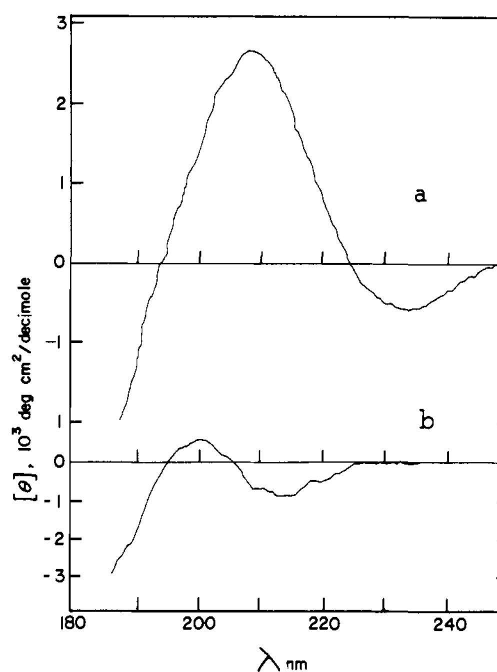

Figure 3. Circular dichroism of glucuronic acid, 14 mg/ml, (a) at pH 2.5 and (b) at pH 6.9 (glucuronate).

orientation of substituent groups, has not been determined. Urea (8 M) changes neither the CD nor ORD of dilute hyaluronate solutions,[9] although urea induced changes have been reported for concentrated solutions where aggregation may occur.[10]

It is now possible to measure CD in the vacuum ultraviolet region and results on some unsubstituted monosaccharides[11] and a polysaccharide, ι-carrageenan,[12] have been reported. We present here the first VUCD study of a glycosaminoglycan. Of importance in this study are the location of the optically active transitions above 170 nm in glucuronic acid and *N*-acetylglucosamine, an assignment of the optical features of hyaluronic acid to monomeric transitions, and the connection between those optical features and the conformation of hyaluronic acid in solution.

Experimental Section

The sodium salt of hyaluronic acid, prepared from rooster comb, was obtained from Biotrics, Inc. The protein content of the sample was less than 0.1%. Research grade D-glucuronic acid and *N*-acetyl-D-glucosamine were obtained from Sigma Chemical Co. The polymer concentration was calculated on the basis of hexuronic acid determination according to a method described previously.[13]

A Cary 60-6001 spectrometer was used to measure the CD spectra above 200 nm of an aqueous solution of 0.015 M sodium glucuronate during titration wih 0.5 M NaOH and 0.5 M HCl. Smooth line drawings of those spectra are reported. All other CD measurements were made on a VUCD spectrometer which has been described previously.[14-17] The instrument was operated with a spectral width of 1.6 nm, a time constant of 30 s, and a scan rate of 0.5 nm/min. Commercial fused silica cells with path lengths of 100 and 46 μ were used, as well as a cell having a nominal path length of 25 μ made up of two CaF_2 disks separated by aluminum spacers. Hyaluronic acid (pH 2.5) is highly viscous and flows very slowly into the small path length cells. Films were cast on 1-mm thick CaF_2 disks by evaporation of the solution to dryness in a nitrogen-filled glove bag at 23 °C. Rotation of the films about the optical axis had no effect on the signals reported here, and there was no indication of flattening of the CD bands. The molar ellipticity, [θ], of the polysaccharide is reported on the basis of the disaccharide molecular weight. The results obtained with the VUCD instrument are presented here as direct tracings of recorded spectra.

Results and Discussion

Glucuronic acid and sodium glucuronate. Figures 1 and 2 show the pH dependence of the CD of an aqueous solution of glucuronic acid above 200 nm. The CD of the acid and anion in this wavelength region have been reported previously.[8,18,19] The acid displays a negative band at 233 nm and a positive band at 207 nm. As the pH is raised to 6.9 the 233-nm band gradually disappears. The anion is characterized by a negative band at 214 nm and a crossover at 206 nm. There was a tendency for the Cary instrument to display a positive extremum near 204 nm at pH 6.9, but our data on the VUCD instrument (see below) indicate that the extremum is not reached until 200 nm. The CD at pH 11 is no different from the CD at pH 6.9.

Figure 3a shows the CD of an aqueous solution of glucuronic acid (pH 2.5) and Figure 3b that of an aqueous solution of sodium glucuronate (pH 6.9) obtained on the VUCD spectrometer with a 100 μ path length cell. These data place the positive band in the anion at 200 nm. The CD data from 185

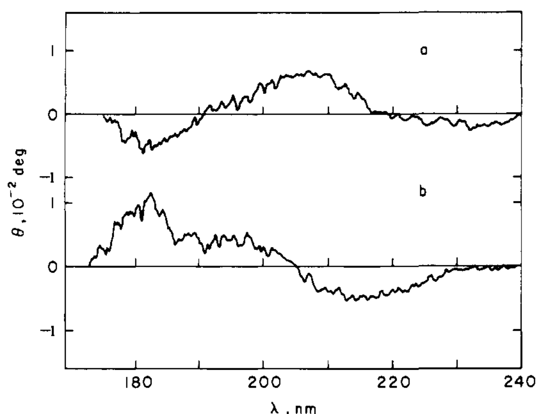

Figure 4. Circular dichroism film spectrum of (a) glucuronic acid and (b) sodium glucuronate.

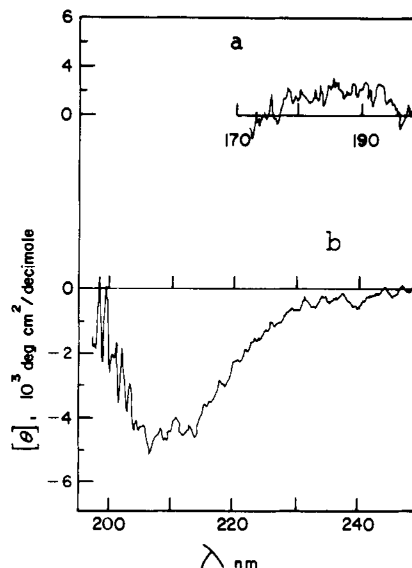

Figure 5. Circular dichroism of *N*-acetylglucosamine, 6 mg/ml.

to 200 nm reveal that the crossover in both the acid and anion is at 194–195 nm, below which there is substantial negative ellipticity. At 185 nm the negative ellipticity is significantly greater in magnitude in the anion than in the acid. Spectra were also obtained with cells of 46 and 25 μ path length. The signal-to-noise ratio below 185 nm was not good in those spectra and they are not presented here, but the data did suggest that the extremum of the high-energy negative band is centered near 182 nm in both the acid and anion forms and that the ellipticity decreases again in magnitude below 182 nm.

VUCD spectra were also obtained with films cast from aqueous solution; the results are presented in Figures 4a (glucuronic acid) and 4b (sodium glucuronate). The film spectrum for glucuronic acid is identical with the solution spectrum above 185 nm; at lower wavelengths a negative band appears at 182 nm which tends to confirm our data in solution measured with the 25 and 46 μ path length cells. The film spectrum of the anion is the same as the solution spectrum above 200 nm, but below 200 nm, where the solution spectrum becomes negative, the film spectrum remains positive down to the cutoff near 174 nm. The anion film spectrum indicates a positive band at 182 nm and another near 197–198 nm.

From Figures 3 and 4 we conclude that there is an optically active transition at 182 nm in glucuronic acid and in glucuronate, the rotational strength of which is negative in solutions of both species and in films of the acid; in films of the anion its rotational strength is positive. The location of that band is close to what is expected for a carboxyl $\pi-\pi^*$ transition. Snyder et al.[20] observed a strong negative CD band near 170 nm in hexafluoro-2-propanol solutions of a series of zwitterionic alkyl amino acids, and the assignment of that band to a carboxyl $\pi-\pi^*$ transition is straightforward. The shift to 182 nm in our spectra can be accounted for in terms of the difference in solvent. We cannot rule out, however, the possibility that the 182-nm band we observe reflects a ring transition. The VUCD of glucose and other monosaccharides[11,21] exhibits weak ellipticity near 180 nm, and Balcerski et al.[12] observed a negative 180-nm band in ι-carrageenan. It is also possible that our 182-nm band has contributions from both a carboxyl $\pi-\pi^*$ transition and a ring transition.

In the protonated form the 182-nm band is negative in solution and in films; in the anion it is negative in solution but positive in films. X-ray crystal studies of salts of glucuronic acid[22,23] indicate that intermolecular interactions as well as carboxyl–cation interactions are large enough to account for strong perturbations of the glucuronate chromophore, certainly large enough to explain the change in the sign of the CD in the film relative to the solution.

The CD features above 190 nm must be assigned to the carboxyl chromophore.[11,21] The 233- and 207-nm bands of the acid form have been assigned[24-27] to an n–π^* transition, and the appearance of two bands is explained as arising from different rotational isomers.

According to the interpretation of Morris et al.[27] the CD features of the anion above 190 nm (Figures 3 and 4) are also to be attributed to the existence of two rotational isomers, with the negative charge of the anion causing blue shifts relative to the acid.

An alternative explanation of the anion spectrum can be developed from the fact that the crossover near 206 nm nearly coincides with the maximum in the acid spectrum. This makes possible an assignment based on the existence of only one rotational isomer in the anion, the CD of which represents an n–π^* band which has a sigmoidal band shape. Most likely the coincidence of the crossover in the anion spectrum and the maximum in the acid spectrum is fortuitous and the explanation of the spectra given by Morris et al.[27] is correct.

We therefore interpret the rather complex CD spectra of glucuronic acid and sodium glucuronate above 175 nm in terms of two monomeric transitions, one located at 182 nm which is probably a carboxyl $\pi-\pi^*$ transition, and the other a carboxyl n–π^* transition, which gives rise to two n–π^* bands because of the presence of two rotational isomers.

N-Acetylglucosamine. Figure 5 shows the CD above 175 nm of an aqueous solution of *N*-acetyl-D-glucosamine obtained with path lengths of 100 (curve b) and 46 μ (curve a) on the VUCD spectrometer. The negative CD band near 210 nm has been reported previously.[8,28,29] We find a crossover at 197 nm and a positive band near 188 nm having an ellipticity of approximately +2000 deg cm^2 $dmol^{-1}$. At 175 nm the ellipticity is approximately zero. We found the CD of *N*-acetylglucosamine insensitive to pH changes.

The positive band at 188 nm can be assigned to the amide $\pi-\pi^*$ transition. Amide absorption maxima are typically near 190 nm, and the alternative assignment to a ring transition is counterindicated by the absence of CD in that region in unsubstituted monosaccharides.[11,21] The negative band must be assigned to an amide n–π^* transition. The minimum of that band is not sharp. Figure 5b shows a typical scan; signal averaging would produce a band shape with a minimum definable only to within a few nm, i.e., 208–210 nm. Since "unsolvated" amides typically have CD bands at higher wavelengths, e.g., at 222 nm in α-helical polypeptides, we can conclude that the amide group in *N*-acetylglucosamine is highly solvated.

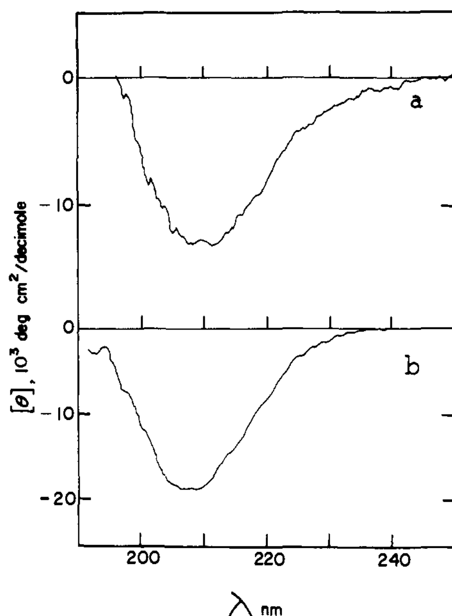

Figure 6. Circular dichroism of hyaluronic acid (a) at pH 2.5 and (b) at pH 6.9 (hyaluronate), 100 μ path length, 3 mg/ml.

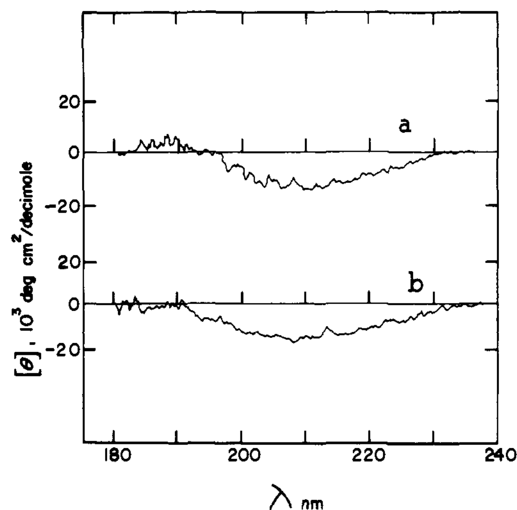

Figure 7. Circular dichroism of hyaluronic acid (a) at pH 2.5 and (b) at pH 6.9 (hyaluronate), 46 μ path length, 3 mg/ml.

Hyaluronic acid and sodium hyaluronate. Figure 6a shows the CD above 195 nm of an aqueous solution of hyaluronic acid (pH 2.5) and Figure 6b that of an aqueous solution of sodium hyaluronate (pH 6.9), both measured on the VUCD spectrometer with a 100 μ path length cell. In the acid there is a strong negative band near 210 nm, and the signal crosses the baseline near 195 nm. We often observed positive pen deflections below 195 nm when the 100 μ cell was used; at times the deflection was equal in magnitude to the negative band. At lower concentrations of hyaluronic acid in the 100 μ cell the large positive pen deflections below 195 nm were not observed. At pH 6.9 (curve b) the negative 208-210-nm band is somewhat larger than at low pH, and the signal does not cross the baseline above 190 nm, in contrast with the acid CD. No positive pen deflections were ever observed below 195 nm with hyaluronate in the 100 μ cell, in contrast with the acid. Also, the negative CD signal near 235 nm in hyaluronic acid is not observed in hyaluronate.

Figure 7a shows the CD above 180 nm of an aqueous solution of hyaluronic acid (pH 2.5) measured on the VUCD spectrometer with a 46 μ cell, and Figure 7b shows the CD of an aqueous solution of hyaluronate (pH 6.9). The shorter path length allowed further penetration into the vacuum ultraviolet region. The CD of the acid shows a positive band centered near 188 nm, then drops back to the baseline at 180 nm. There is no indication of a positive band in the polyanion in the region from 180 to 190 nm.

The CD of hyaluronic acid has been previously reported by Stone[29,30] and by Chakrabarti and Balazs.[8] Our data agree with the earlier reports except that we find the 188 nm positive band in the acid to be rather weak, and we see no negative ellipticity in the polyanion from 180 to 190 nm.

The weak negative ellipticity near 233 nm in the acid (Figure 6a) likely has its origin in the glucuronic acid moiety. If it represents a second rotational isomer, as discussed above, its appearance in the polymer CD indicates that the same configurational preference of the carboxyl group that exists in the monomer is maintained in the polymer. At pH 6.9 the 233 nm ellipticity disappears in the polyanion just as in the monomeric anion.

The 210-nm polymer band must have parentage in either the amide n-π* transition of the *N*-acetylglucosamine moiety or the carboxyl n-π* transition of the glucuronic acid moiety, or both. The pH dependence of the intensity of the polymer band indicates that part of that intensity, at least, comes from the latter.

Our monomer spectra correspond to equilibrium mixtures of α and β anomers, whereas only β linkages exist in the polysaccharide, so that one cannot expect the polymer cd to be strictly an additive combination of our monomer CD spectra. On the other hand, the CD near 210 nm of glucuronic acid, α-D-glucopyranosiduronic acid, and β-D-glucopyranosiduronic acid are all rather similar.[19] Similarly, the CD of *N*-acetylglucosamine, methyl 2-acetamido-2-deoxy-β-glucopyranoside, and methyl 2-acetamido-2-deoxy-β-glucopyranoside are also similar.[28] The intensities of the 210-nm polymer bands, approximately −14 000 and −18 000 deg cm^2 $dmol^{-1}$ for the acid and anion forms, respectively, are too great to be accounted for by a simple additivity of monomeric spectra, even after consideration of the effect of the anomeric equilibria on the monomer data. Since the observed nonadditivity is outside the limit of error in our determination of the polysaccharide concentration, we conclude that the nonadditivity is significant. Earlier evidence for the nonadditivity of the CD of hyaluronic acid was obtained in a study of its tetra- and hexasaccharide degradation products;[8] the magnitude of the CD at 210 nm, per disaccharide, is larger in the polysaccharide than in the tetramer or hexamer.

Several types of interaction could account for the nonadditivity. The configuration of the sugar ring and/or its hydroxyl groups might change in the presence of neighboring sugar rings. The polarizability and static field contributions to the optical activity of the n-π* transitions of substituent groups would both be affected. Another possibility is that the orientation of the acetamido and/or carboxyl groups is affected by neighboring residues, which would also affect the rotational strength of their optical transitions. These two types of mechanism include perturbations by neighboring residues. It is difficult to envision how nonnearest neighbor interactions could give rise to the nonadditivity. The interactions which are dominant in helical polypeptides, for example, are probably not operative here. Some structural element involving the close contact of nonnearest neighbors may be giving rise to the nonadditivity.

In a recent NMR study Darke et al.[6] concluded that the hyaluronate chain in solution can be described as having two domains, one of which is flexible and the other stiff. Whether the nonadditivity in the optical property has any relation to the

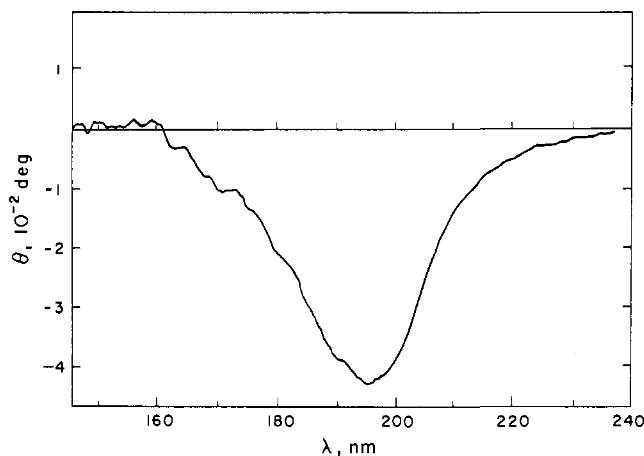

Figure 8. Circular dichroism of a sodium hyaluronate film.

presence of stiff domains in the chain or reflects purely localized perturbations cannot be determined from our data. It is possible that the structural elements of the stiff domains are responsible for the nonadditivity of the CD intensity near 210 nm.

We assign the positive 188-nm CD band observed in hyaluronic acid to the π–π^* transition of the amide group of *N*-acetylglucosamine. That band is similar in position and approximate magnitude to the band we observe in *N*-acetylglucosamine. The negative dichroism we find in glucuronic acid at 182–190 nm is apparently not strong enough to cancel the amide π–π^* band. It was previously noted that the negative band at 182 nm in glucuronate (pH 6.9) is stronger than in the acid. We conclude that no positive 188-nm band is observed in hyaluronate because it is canceled by the negative glucuronate band. Thus the appearance of the positive 188-nm band in hyaluronic acid at low pH is attributable to protonation of the carboxyl groups.

Since the pH dependence of the intensities of the 188- and 210-nm bands both can be explained in terms of protonation of the carboxyl groups, conformational changes in the chains are probably not responsible for the CD changes. Although it is well known that the rheological properties of hyaluronic acid change significantly as a function of pH,[3,4] CD may not be a direct probe for those rheological changes. It is interesting to compare this situation with the case of polypeptides, in which it has been established that protonation is not the cause of the CD dependence on pH, but rather that the CD changes directly reflect true conformational changes.

Figure 8 shows the CD of a film of sodium hyaluronate cast from aqueous solution. A negative band appears near 194 nm, which we assign to the π–π^* band of the acetamido group. The n–π^* bands are hidden in the long-wavelength tail of the band. It is also quite possible that the carboxyl π–π^* band located near 182 nm is hidden in the short-wavelength tail of the band.

From an estimate of the amount of the polysaccharide deposited as film, and assuming uniform film thickness, the molar ellipticity at 194 nm in Figure 8 can be calculated to be $-2.0 \times 10^4 \pm 0.2 \times 10^4$ deg cm^2 dmol^{-1}. The absorption spectrum of the same film, not shown here, displayed a strong absorption near 195 nm in the form of a plateau before increasing further at lower wavelengths. The coincidence of the CD band maximum and strong absorption confirms the assignment to a π–π^* transition.

The CD of the π–π^* transition is much greater in the hyaluronate film than in solution. The large rotational strength must reflect a much decreased rotational flexibility of the acetamido group in the film. Since our film was prepared from the same type of hyaluronate solution and approximately in the same way as the films studied recently by x-ray diffraction,[2b] it is quite likely that in our films the hyaluronate chains are in the left-handed fourfold helical structure reported in the diffraction study. In that structure the acetamido group takes part in an intramolecular hydrogen bond with the carboxyl group of the neighboring residue. We believe that it is the formation of this hydrogen bond which accounts for the decreased rotational mobility of the acetamido group and thereby gives rise to the large rotational strength of the acetamido π–π^* transition in our films.

If a negative CD band near 198 nm is indicative of an intramolecular hydrogen bond between the acetamido and carboxyl groups, it is interesting to note that such a band also appears in an aqueous solution of hyaluronic acid (pH 2.5) 5% in ethanol.[9] The absence of such a band in aqueous solutions reflects the absence of such intramolecular hydrogen bonds, indicating a higher rotational freedom of the acetamido group. Furthermore, whatever the structural features are of the stiff domains in solution indicated by NMR[6] the absence of the negative 198-nm CD band in solution shows that under our experimental conditions the acetamido groups have substantial rotational freedom and are likely not involved in stabilizing the stiff domains.

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