ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 265, No. 2, September, pp. 484-495, 1988

Self-Association of Hyaluronate Segments in Aqueous NaCl Solution

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Received January 15, 1988, and in revised form May 9, 1988

The potential for self-association by hyaluronate (HA) chains in 0.15 M NaCl was investigated, using low molecular weight HA segments as a model system. HA segments were derived from the polymer by controlled enzymatic digestion, and purified by gel filtration chromatography. Seven samples of narrow molecular weight distribution were analyzed by sensitivity-enhanced polyacrylamide gel electrophoresis, and found to have the following weight-average numbers of repeating disaccharide units: A, 90; B, 51; C, 38; D, 31; E, 23; F, 18; G, 13. The segment preparations were studied in 0.15 M NaCl by capillary viscometry, low angle laser light scattering, and circular dichroism spectroscopy. The data indicate concentration-dependent intermolecular association of short segments, and a capability for intramolecular association (hairpin formation) by larger HA segments.

Hyaluronic acid (HA)[2] is a high molecular weight glycosaminoglycan with a wide distribution in connective tissues of the body. It has a linear copolymeric structure, composed of alternating 1- and 3-linked 2-acetamido-2-deoxy-β-D-glucose (GlcNAc) and 1- and 4-linked β-D-glucuronic acid (GlcUA) residues (1). In certain liquid connective tissues such as the eye vitreous, HA is thought to act as a molecular filter, shock absorber, and support structure for collagen fibrils (2). These functions are further proposed to depend on the existence of an HA network formed by fleeting interactions between segments of neighboring polymer chains (3).

It is now well established that HA chains can dimerize under certain conditions. A double helical structure has been observed (4) in oriented films of HA, under admittedly unusual counterion conditions (e.g., Rb^+, Cs^+, mixed K^+, H^+). Solutions of HA form stiff gels at pH approximately 2.5, in mixed ethanol-water solutions with added NaCl (5). The gel structure has been shown to involve interpolymeric interactions, which can be disrupted by the addition of HA fragments (3, 6) or hydrogen bond-breaking solvents (7). The interactions were further shown by circular dichroism studies to be dimeric in nature (8).

A clear demonstration of self-association under physiological ionic conditions has been more elusive. Viscoelasticity studies suggest a weak and dynamic self-association in NaCl solution (6, 9). Some light scattering data also indicate the existence of interactions between polymers (10-12), but have proven difficult to reproduce (13). The bulk of physical studies on HA have been interpreted on the basis of random or worm-like coils of moderate stiffness, and have not required the consideration of intermolecular interactions (14-16).

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[2] Abbreviations used: HA, hyaluronic acid; GlcNAc, 2-acetamido-2-deoxy-β-D-glucose; GlcUA, β-D-glucuronic acid; GAG, glycosaminoglycan; SEPAGE, sensitivity-enhanced polyacrylamide gel electrophoresis; LALLS, low angle laser light scattering.

0003-9861/88 $3.00

We have considered that one difficulty in the interpretation of most such studies involves the use of polymeric samples of high molecular weight. Thus chain–chain interactions may occur both intermolecularly and intramolecularly (between segments of a single chain). The predominate type of association may depend on a number of variables, including the method of sample preparation. An alternative approach is to choose a model system incapable of forming both types of interactions.

In the present study, a series of short HA segments are analyzed in physiological (0.15 M) NaCl solution. The absolute molecular weight of each segment sample was established by a recently developed electrophoretic method, and compared with the apparent molecular weight in solution, determined by physical techniques. By both viscometric and light scattering analyses, the HA segments show no evidence of intermolecular association in the limit of zero concentration. The concentration dependence of these data, in contrast, provide evidence for weak intermolecular interactions. Intramolecular interactions can also occur in HA segments of sufficient length. Additional circular dichroism studies suggest that association is correlated with a subtle change in the environment of the acetamido group.

MATERIALS AND METHODS

Materials. Human umbilical cord HA was grade I material from Sigma Chemical Co. Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35, 4883 units/mg) was purchased from Cooper Biomedical Corp. DEAE-Sephacel anion-exchange resin was obtained from Pharmacia AB. Bio-Gel P-200 (100–200 mesh) gel filtration medium, all reagents for polymerization of electrophoretic gels, and the silver staining reagents were obtained from Bio-Rad Laboratories. Alcian blue 8GS was obtained from Fluka Chemical Corp. Membrane filters of 0.2 μm nominal pore diameter (Gelman acrodisc) were obtained from Fisher Scientific. Water was deionized and glass distilled. All other chemicals were ACS reagent grade quality.

Purification of HA. Many commercial samples of umbilical cord HA (including that used in the present study) contain 5–25% sulfated glycosaminoglycan (GAG). The sulfated GAG was removed by a one-step ion-exchange purification method (J. D. Gregory, personal communication). HA was dissolved in 0.3 M NaCl at a concentration of 2.5 mg/ml. The solution, containing a total of 500 mg HA, was passed through a 2 × 12-cm column of DEAE-Sephacel equilibrated in the same solvent. The flow rate of the viscous HA solution was increased to approximately 20 ml/h, by application of reduced pressure to the receiving flask. Under these conditions, sulfated GAG remains bound to the resin, and HA passes through. The sulfated GAG content after purification was checked by sensitivity-enhanced polyacrylamide gel electrophoresis (SE-PAGE), and found to be less than 1% of the HA (w/w), which is the approximate detection limit by this method.

The protein content of the HA at this point was found to be 1.5%, using the Bio-Rad Protein Microassay (Bio-Rad Laboratories), with bovine serum albumin as standard. Reduction of the protein contamination was performed by a procedure similar to that of Balazs (17). The HA sample was made 1.7 M in NaCl by addition of dry NaCl. An equal volume of chloroform was added, and the mixture was shaken overnight. The aqueous and organic layers were separated, and the aqueous fraction was reextracted with fresh chloroform. The sample was then adjusted to pH 4.5 with 0.1 M HCl, and extracted twice with chloroform. The purified HA solution was dialyzed exhaustively against H_2O, lyophilized, and stored desiccated at 4°C. The final protein content was 0.7%.

Enzymatic digestion. A 4 mg/ml solution of HA in Buffer A (150 mM NaCl, 100 mM CH_3COONa, 1 mM Na_2EDTA, pH 5.0) was prepared by slow stirring at 4°C. A total volume of 72 ml was divided into three equal aliquots. The aliquots were preincubated for 0.5 h at 37°C, with slow stirring. Bovine testicular hyaluronidase was added to each of the three samples as 1-ml aliquots containing 6000 units of enzyme in Buffer A. Digestion was allowed to proceed for 15, 30, and 45 min for the three samples, respectively. Digestions were terminated by heating in a boiling water bath for 10 min. The samples were cooled to room temperature and centrifuged at 15,000*g* for 10 min. The three supernatants were pooled to provide a broad mixture of HA fragment sizes.

Gel filtration chromatography. A 2.5 × 195-cm column of Bio-Gel P-200 (100–200 mesh), equilibrated in 0.5 M NaCl, was employed for fractionation of the pooled HA digest. The HA sample was made 0.5 M in NaCl by addition of dry NaCl. Aliquots containing approximately 45 mg of HA were chromatographed at a flow rate of 12 ml/h. Fractions of 7 ml were collected and analyzed for uronic acid content by the automated carbazole method of Balazs *et al.* (18). A total of five sample aliquots were chromatographed in order to obtain sufficient material.

The fractions obtained from the first chromatographic run were combined into eight pools differing in average HA molecular weight. Selection of the fractions for pooling was based on the average chain length of HA species present, as determined by gel electrophoresis (see below). Individual fractions were analyzed without removal of NaCl. By this screening procedure, it was possible to maintain a low degree of molecular weight heterogeneity in each pool and to subsequently combine fractions containing only corresponding species from separate chromatographic runs. The eight pools have been designated A through H, corresponding to HA segments of decreasing molecular weight. Pool H, containing low molecular weight oligosaccharides, was not further analyzed. The remaining HA segment samples were dialyzed exhaustively against H_2O, lyophilized, and stored desiccated at 4°C.

Polyacrylamide gel electrophoresis. Sensitivity-enhanced polyacrylamide gel electrophoresis was used to determine the molecular weight of HA segment samples. The polyacrylamide gel composition was 10% acrylamide, 0.33% bisacrylamide, 0.1 M Tris-borate, 0.001 M Na_2EDTA, pH 8.3, prepared as previously described (19). Slabs of 32 × 14 × 0.15 cm were employed. SE-PAGE electrophoresis and alcian blue-silver staining of gels were performed as described elsewhere (20).

Purified HA segment samples were dissolved in H_2O at a concentration of 0.1 mg/ml. For photographic purposes, sample loads of approximately 2 μg were used. For more precise determination of molecular weight, sample loads of 0.1–1.0 μg were employed. Gels were scanned at 615 nm on a Kontes Model 800 densitometer. The densitometric patterns were used to calculate number- and weight-average degree of polymerization (N_n, N_w) for each sample as previously described (20). The corresponding average molecular weights (M_n, M_w) were calculated on the basis of a disaccharide residue weight of 401, for the sodium salt of HA.

Viscometry. HA segments were dissolved in 0.15 M NaCl at a concentration of 8 mg/ml and dialyzed exhaustively against the same solvent. Samples were filtered through a 0.2-μm membrane, and an aliquot was removed for concentration determination by the automated carbazole procedure. Viscosity measurements were made using a Cannon-Ubbelohde semimicro capillary dilution viscometer (type 50), at a constant temperature of 25.0°C. Reduced viscosity (η_{sp}/c) was determined for each sample at a minimum of 4 concentrations using dialysate as diluent, and extrapolated to zero concentration to obtain the intrinsic viscosity, $[\eta]$. No correction was made for solution density. The Huggins' parameter, k', was calculated as the slope of the same plot divided by the square of the intrinsic viscosity.

Low angle laser light scattering (LALLS). HA segment samples were dissolved in 0.15 M NaCl at a concentration of 4–8 mg/ml, and dialyzed exhaustively against the same solvent. The refractive index increment (dn/dc) at 633 nm was determined by using a Chromatix KMX-16 laser differential refractometer (LDC/Milton Roy). Sample at four different concentrations was filtered through a 0.2-μm membrane directly into the cell. Concentration was subsequently measured by automated carbazole assay. The value of dn/dc for HA segment sample C was found to be 0.153, in agreement with published data for high molecular weight HA (12, 13, 21). We have assumed this value to be the same for the remaining HA segments. The optical constant K was therefore 1.71×10^{-7} mol cm^2/g^2.

Light scattering measurements were performed on a Chromatix KMX-6 laser light scattering photometer (LDC/Milton Roy). Samples were filtered on-line through a 0.2-μm membrane, and passed through the 15-mm-path-length scattering cell at a flow rate of 0.1 ml/min. Scattered light was detected using the 6–7° annulus (4.86° effective average scattering angle). The scattered light intensity was quantitated as the excess Rayleigh factor $\tilde{R}_\theta$, defined as the difference in Rayleigh factors of solution and solvent. The solution Rayleigh factors ranged in magnitude from twice to approximately 30 times that of the solvent. Sample concentrations (c, in g/ml) were determined by automated carbazole assay of aliquots collected from the scattering cell outlet after the photometer reading had stabilized. Kc/R_θ was thus determined at a minimum of 4 concentrations for each sample, and extrapolated to zero concentration. The reciprocal of the y-intercept yielded M_w, and one-half the slope yielded the second virial coefficient, A_2.

Spectroscopic analysis. Circular dichroism (CD) spectra were measured at room temperature, using a Jasco J-500A recording spectropolarimeter with a Jasco IF-500 interface and an IBM PC. The instrument was calibrated at 192.5 and 290.5 nm with d-10 camphorsulfonic acid (22). Single-scan spectra were generally recorded under the following conditions: 0.02-cm-path-length quartz cell, 2 mdeg/cm sensitivity, 1 nm/min scan speed, 1 nm spectral band width, 16 s time constant. The signal-to-rms noise ratios were approximately 100:1 at 210 nm, and 10:1 at 196 nm, without spectral smoothing.

Molar ellipticity values ($[\theta]$, in units of deg cm^2 dmol^{-1}) were calculated on the basis of the disaccharide residue concentration, determined from the absorption spectrum of each CD sample. The absorption spectra were recorded using a Varian 2300 spectrophotometer. The molar extinction coefficient $\epsilon_{188} = 1.1 \times 10^4$ liters cm^{-1} mol^{-1} was previously determined (23). The same data, measured at a 1 nm spec-

tral band width, yield a value of $\epsilon_{200} = 4.74 \times 10^3$, useable when solvent absorbance precludes acquisition of data at 188 nm.

RESULTS

Isolation and electrophoretic characterization of HA segments. Limited degradation of NaHA by bovine testicular hyaluronidase was used to prepare a homologous series of fragments 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. The fragment mixtures prepared under three different conditions of digestion were pooled to yield a broad molecular weight distribution. The pooled digest was fractionated by gel filtration chromatography on Bio-Gel P-200, eluted with 0.5 M NaCl. Seven pools, designated A through G, in order of decreasing molecular weight, were collected for further analysis. The elution positions of the HA segment pools are given in Table I.

HA segment samples were analyzed by SE-PAGE to determine molecular weight distributions and averages. Figure 1 shows the appearance of an SE-PAGE separation for the HA segment samples. Each sample exhibited a ladder-like series of bands. The bands of greatest mobility (bottom of the gel) correspond to species with the lowest molecular weight. Adjacent bands correspond to HA fragments differing in chain length by one disaccharide unit of the repeating structure (19, 24). Band identification was accomplished by coelectrophoresis of a purified oligosaccharide (n = 11) and the original digest mixture, which serves as a band counting aid. The purified oligosaccharide had been previously isolated and characterized (24).

The molecular weight distributions of the HA segment samples were characterized by densitometry of SE-PAGE gels. Each segment was studied at several sample loads to eliminate stain saturation artifacts, and was electrophoresed for a time period appropriate for maximum band separation. The range of species present, the weight-average and number-average degrees of polymerization, the corresponding molecular weight averages, and the polydispersity index were determined for each sample. Results are given in Table I. All samples show extremely low polydispersity. The set spans an approximately sevenfold range in molecular weight (approximately 5000–36,000).

Viscometry. The limiting viscosity number was determined for segments A–F in 0.15 M NaCl, at 25°C. Figure 2 shows the viscometric data. The intrinsic viscosity values, obtained as the zero concentration intercept of the linear least-squares fit for each segment, are given in Table II. Figure 3 shows the relationship observed between the intrinsic viscosity and weight-average molecular weight determined by SE-PAGE. Two additional data points included in Fig. 3 were obtained in previous studies of low molecular weight HA seg-

TABLE I

SE-PAGE CHARACTERIZATION OF HYALURONATE SEGMENTS

Sample	K_{av}[a]	Range (N)[b]	$\bar{N}_W$[b]	$\bar{N}_N$[b]	$\bar{M}_W$	$\bar{M}_N$	$\bar{M}_W/\bar{M}_N$
A	0–0.10	49–130	90.0	84.7	36,100	34,000	1.06
B	0.11–0.17	33–70	50.5	48.6	20,300	19,500	1.04
C	0.18–0.23	32–45	37.5	37.2	15,000	14,900	1.01
D	0.24–0.34	25–37	30.6	30.3	12,300	12,200	1.01
E	0.35–0.46	19–27	23.1	22.9	9,260	9,180	1.01
F	0.47–0.59	15–20	17.5	17.3	7,020	6,940	1.01
G	0.60–0.70	12–15	13.4	13.3	5,370	5,330	1.01

[a] K_{av} = (elution volume − void volume)/(total bed volume − void volume).

[b] Number of repeating disaccharide units.

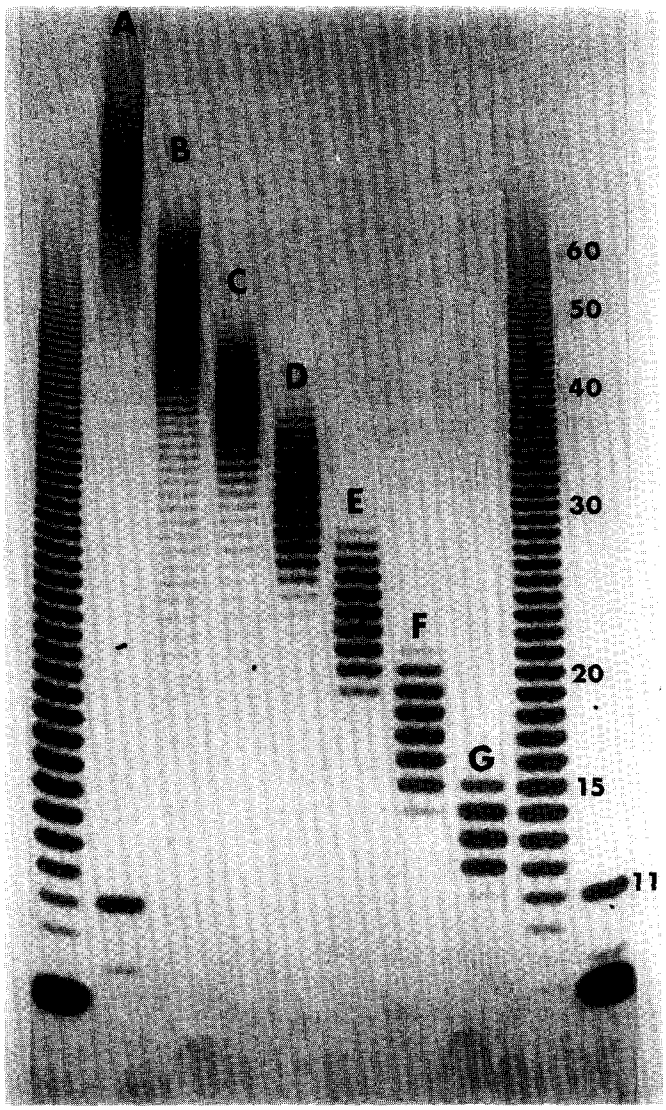

FIG. 1. Sensitivity-enhanced polyacrylamide gel electrophoresis (SE-PAGE) of HA digest and purified HA segments. From left: Lanes 1 and 9, unfractionated HA digest; lanes 2-8, HA segment preparations A-G. An HA oligosaccharide of 11 repeating disaccharide units was added to lanes 2 and 10. This allowed identification of all other HA bands in the gel according to the number of disaccharide units, as indicated. Bromphenol blue tracking dye was added to lanes 1 and 10.

ments (25). The relation $[\eta] = 6.54 \times 10^{-4}$ $M^{1.16}$ was observed to fit the data. This expression is in reasonable agreement with the findings of Cleland (16), who observed $[\eta] = 2.8 \times 10^{-3}$ M for low molecular weight HA samples in 0.2 M NaCl. In the latter study, molecular weight was determined by sedimentation equilibrium, and therefore potentially complicated by intermolecular association equilibria. The observed similarity between the present data and those of Cleland indicates that the zero concentration limit of sedimentation data yields the same molecular weight value as that obtained by SE-PAGE.

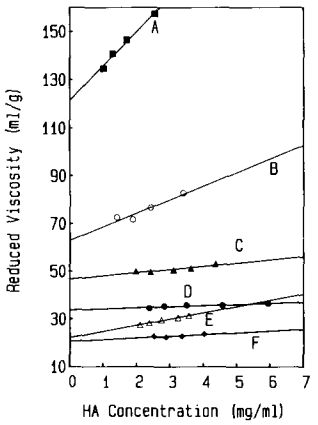

FIG. 2. Analysis of HA segments in 0.15 M NaCl by capillary viscometry.

Table II also reports the Huggins' constant, k', determined from the slope of the reduced viscosity plot for each sample. Shimada and Matsumura (26) previously reported k' values for more polydisperse samples of HA in 0.1 M phosphate buffer at 37°C. Our data yield significantly higher k' values. These data suggest the existence of intermolecular association between HA segments in 0.15 M NaCl, leading to changes in chain conformation and/or solvation. Under these conditions, the reduced viscosity increases more rapidly with concentration than expected (27).

Low angle laser light scattering. More direct evidence for the existence of an association equilibrium between HA segments in 0.15 M NaCl was obtained by LALLS. Figures 4 and 5 show the LALLS data for HA segments B-E. The reciprocal of the zero concentration intercept in each plot yields the weight-average molecular weight. These values are given in Table II. Good agreement was observed between the molecular weights obtained by light scattering and electrophoretic methods. At the limit of zero concentration, no evidence for intermolecular association was observed by either LALLS or viscometric studies.

The concentration dependence of the light scattering function does provide evidence for self-association. The apparent second virial coefficient, A_2, may be obtained from the slope of the LALLS data plot for each sample. For high molecular weight ($M_r > 10^6$) NaHA in aqueous salt solutions, A_2 is generally found to be in the range $2.5\text{-}3.5 \times 10^{-3}$ (21). For HA segment B, we observe a similar value of 2.9×10^{-3}. The dependence of A_2 on molecular weight, due to the excluded volume contribution, should theoretically result in a higher value of A_2 for HA segments, relative to the polymer. Thus the measured A_2 is lower than expected for HA segment B. For HA segment C, a more remarkable decrease in A_2 is noted, to 0.4×10^{-3}. Segments D and E show large negative values of A_2. These data are a clear indication of intermolecular association. The negative slope results from the dissociation of interacting species with decreasing concentration. At the limit of zero concentration, only the monomeric species exists. The transition from negative to positive second virial coefficients observed with increasing HA chain length is attributed to

TABLE II

CHARACTERIZATION OF HYALURONATE SEGMENTS BY VISCOMETRY AND LIGHT SCATTERING

Sample	M_W (PAGE)	$[\eta]$	k'	M_W (LS)	A_2 ($\times 10^3$)
A	36,100	122	0.97	—	—
B	20,300	63	1.4	21,700	2.9
C	15,000	47	0.63	14,200	0.4
D	12,300	34	0.42	14,800	−7.0
E	9,260	22	5.3	7,200	−9.8
F	7,020	21	1.7	—	—

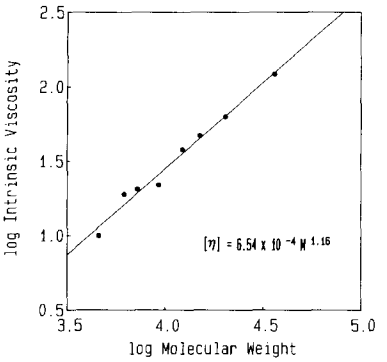

FIG. 3. Relationship between intrinsic viscosity and weight-average molecular weight for HA segments in 0.15 M NaCl. Molecular weights were determined by SE-PAGE analysis.

a growing tendency toward intramolecular association (chain folding) in the longer chains.

Circular dichroism. Figure 6 presents the CD spectrum of HA segment B dissolved in H_2O at a concentration of 2.3 mg/ml. The spectrum is dominated by a negative band centered at 208 nm, attributed to the n-π^* transitions of the acetamido and carboxylate chromophores. The molar ellipticity of that band, calculated on the basis of the repeating disaccharide residue concentration, is $[\theta]_{208} = -10{,}700$ deg cm^2 dmol^{-1}. In the 190-nm region, the π-π^* transition of the acetamido group provides the major influence. The low apparent ellipticity, $[\theta]_{190} = -4000$ deg cm^2 dmol^{-1}, is the result of large additive positive and negative CD contributions, associated with the formation of the β-1,4 and β-1,3 glycosidic linkages, respectively (23). The spectrum of segment B in H_2O is essentially identical with numerous published spectra of polymeric HA (23, 28–30).

The CD spectra of all HA segments were also examined in H_2O, at concentrations of approximately 1.5 and 4 mg/ml. Qualitatively, all of the spectra were similar. Quantitatively, a chain length dependence in the molar ellipticity was noted, especially below 200 nm. Segments of lower

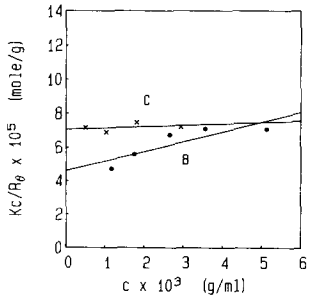

FIG. 4. Low angle laser light scattering analysis of HA segments B (N_w = 50.5) and C (N_w = 37.5) in 0.15 M NaCl.

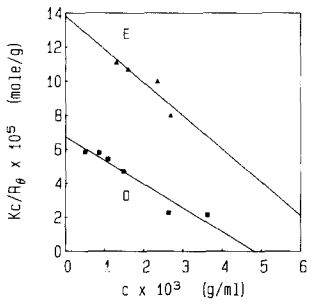

FIG. 5. Low angle laser light scattering analysis of HA segments D (N_w = 30.6) and E (N_w = 23.1) in 0.15 M NaCl.

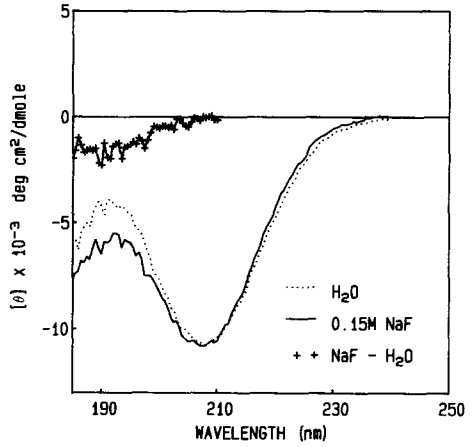

FIG. 6. Circular dichroism spectrum of HA segment B (N_w = 50.5) as a function of added NaF. (· · ·) HA segment B in H_2O at 2.3 mg/ml; (—) HA segment B in 0.15 M NaF at 1.6 mg/ml; (+++) Calculated difference spectrum, obtained as B in NaF minus B in H_2O. Molar ellipticity calculated on the basis of the disaccharide residue concentration.

molecular weight show a larger negative contribution. This phenomenon has been previously noted (23, 28), and attributed to the strongly negative CD contribution of the end sugar residues in fragments with the structure $(GlcUA\text{-}GlcNAc)_n$. Molar ellipticity values, obtained as the means of two or more spectra for each sample, are given in Table III. No concentration dependence was noted in the spectra.

A reversible change in the CD properties of HA segments was observed upon dialysis to 0.15 M NaF or NaCl. Figure 6 compares the CD spectra of segment B in H_2O and 0.15 M NaF solution. In NaF, the negative ellipticity in the 190-nm region is increased, and a minor blue shift in the 208-nm band is observed. The difference spectrum, obtained by subtraction of the H_2O spectrum from that in NaF, shows a negative band centered near 190 nm. Thus, a conformational change affecting the environment of the acetamido group is apparent. This conformational change is proposed to be similar to that found for HA in gels or in solid films (7, 8, 30). In the latter cases, an intense negative CD is observed near 190 nm. The consequence of chain-chain interactions is very much smaller in the present case, presumably as a result of the lower degree of interaction in aqueous salt solution.

The degree of salt-induced conformational change is dependent on the HA segment chain length. Table III presents the data obtained for samples dialyzed into 0.15 M NaF or NaCl, at an approximate concentration of 2 mg/ml. With decreasing chain length, the extent of conformational change is reduced. This is most easily seen in Fig. 7, where the salt-induced change in molar ellipticity at 195 nm is presented as a function of chain length. The choice of 195 nm was dictated by the high absorbance of the NaCl-containing solvent below that wavelength. An HA oligosaccharide of 7 repeating units, available from a previous study (24), showed almost no change in CD. Larger fragments show a progressively greater change in CD, until a sufficient size is reached, and the effect becomes constant. For fragments with a sufficient length, chain folding may be the primary source of the CD change, particularly at the low concentration employed. The possibility of a concentration dependence in the salt-induced CD change was not investigated in the present study, due to limitations in available sample quantity.

DISCUSSION

In the present work, HA segment samples with a low degree of polydispersity were isolated and characterized. The molecular weight of each sample was determined with a high degree of certainty by

TABLE III

CIRCULAR DICHROISM PROPERTIES OF HYALURONATE SEGMENTS[a]

Sample	N_W (PAGE)	$[\theta]_{208}$ (H_2O)	$[\theta]_{208}$ (NaX)[b]	$[\theta]_{195}$ (H_2O)	$[\theta]_{195}$ (NaX)[b]
A	90.0	−10,000	−11,200	−3900	−5900
B	50.5	−10,700	−11,000	−4200	−6500
C	37.5	−10,800	−11,200	−4900	−6400
D	30.6	−10,900	−11,300	−4300	−6700
E	23.1	−10,700	−11,000	−5400	−6400
F	17.5	−10,800	−10,900	−5300	−6600
G	13.4	−10,200	−10,800	−5600	−6600
7	7	−10,100	−10,500	−6100	−6500

[a] Molar ellipticity in units of deg cm^2 $dmol^{-1}$.

[b] NaCl or NaF.

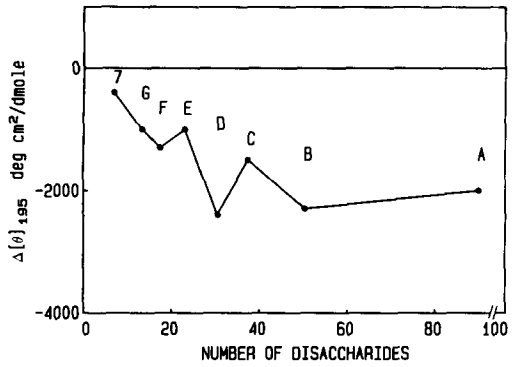

FIG. 7. Salt-induced change in circular dichroism properties for HA segments as a function of chain length. The change in molar ellipticity at 195 nm upon transfer from H_2O to 0.15 M NaCl or NaF is plotted as a function of the weight-average number of repeating disaccharides in each HA segment.

electrophoretic (SE-PAGE) analysis. The series of HA segments forms a model system for the study of interchain association, and other chain length-dependent aspects of HA structure.

Viscometric and light scattering measurements on HA segments in 0.15 M NaCl show no evidence for a stable aggregate (e.g., dimer) in the limit of zero concentration. This conclusion is most directly based on the agreement between the weight-average molecular weight determinations by SE-PAGE and light scattering. Less directly, the viscosity-molecular weight relation observed is in good agreement with that derived by Cleland (16) using sedimentation equilibrium measurements to determine molecular weight for short HA chains. The molecular weight dependence of the intrinsic viscosity, judged by the exponent value of 1.0–1.16 for M, indicates a somewhat stiffened coil structure for the isolated HA chains.

Self-association of HA segments is indicated by the concentration dependence of reduced viscosity and light scattering, and by the circular dichroism results. A model for the behavior of HA segments in 0.15 M NaCl is proposed in Fig. 8, based on the above physical data and published reports from the literature.

Very short oligosaccharide fragments of HA, containing less than approximately 7 disaccharides, are not proposed to undergo self-association. This assertion is based in part on the CD studies of Staskus and Johnson (8). In an acidic ethanol-water solution, polymeric HA is induced to aggregate. A conformational change, monitored as a substantial change in ellipticity near 190 nm, is found to accompany the association. HA segments of sufficient length to undergo intra- or intermolecular dimerization also show this effect. HA fragments of less than 7–9 disaccharides have proven incapable of self-association, even under these extreme solvent conditions. A second line of evidence is based on our previous studies of HA–dye interactions (24). The detection of HA fragments by dye binding in polyacrylamide electrophoretic gels is chain length dependent. It is likely that self-association of HA chains, providing a higher linear charge density, plays an important role in the binding of cationic dyes in the gel matrix. Only fragments containing more than seven disaccharides are capable of dye binding in a 10% polyacrylamide gel.

HA fragments of approximately 7–20 disaccharides are proposed to exist in a monomer–dimer equilibrium. Our samples F and G fall into this category. Sample G (N_w = 13.4), analyzed only by CD, shows a weak salt-induced changed in ellipticity

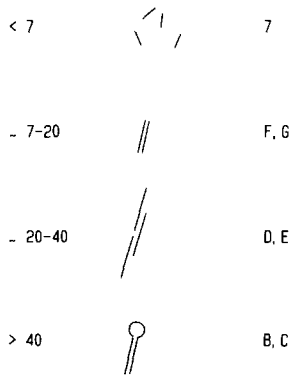

FIG. 8. Model for self-association of HA segments in 0.15 M NaCl, as a function of segment chain length (number of repeating disaccharide units).

near 190 nm, when studied at a concentration of approximately 2 mg/ml. Sample F (N_w = 17.5) shows the same CD effect, to a slightly increased degree. In addition, sample F shows an elevated Huggins' constant, k', in viscometric studies. Both effects are attributed to self-association. Staskus and Johnson (8) found the CD properties of HA species in this size class to be highly concentration dependent in acidic ethanolic solution, in a manner well fit by a monomer-dimer equilibrium. In neutral aqueous solution, the electrostatic repulsion of chains is expected to be minimized by an increased condensation of counterions, accompanying the doubling in linear charge density (31-33). A plausible model for dimer structure would place the two chains out of register by one sugar unit, so that the carboxylate groups are spaced approximately 0.4-0.45 nm apart. This spacing should result in minimum repulsion, but allow counterion condensation, similar in extent to that of the chondroitin sulfates (34). In Fig. 8, the chains of the HA dimer are depicted in a side-by-side arrrangement. The alternative double helical arrangement is also compatible with, but not dictated by, our results. Multichain aggregates are also strictly compatible with the data for samples F and G, but not favored for reasons discussed below in relation to larger HA fragments.

HA segments of 20-35 disaccharides are approximately twice the minimum length required for stabilization of interchain associations. Our samples D and E fall into this category. Sample E (N_w = 23.1) shows a salt-induced increase in CD ellipticity at 195 nm, and an elevated Huggins' constant expected for intermolecular association. The strongly negative apparent second virial coefficient further indicates attractive intermolecular interaction. Sample D (N_w = 30.6) shows similar behavior, except that the Huggins' constant is surprisingly not elevated. Estimation of the apparent molecular weight for these species at a given concentration may be made from the light scattering data, by assuming a minimum positive value of the true second virial coefficient, equal to that of polymeric HA. On this basis, we estimate that species with molecular weights of at least 3-4 times that of a single chain are present. The aggregation beyond the dimer level may be easily accommodated in a two-chain association model, by staggering the chains. Staggered arrangements are possible only when the chain length is at least twice the minimum necessary for association.

HA fragments of approximately 35 or more disaccharides constitute the last group, thought to be capable of chain folding in aqueous solution. This category includes our samples A, B, and the borderline C. Sample A (N_w = 90.0) shows a marked CD change in salt solution. It also shows an elevated Huggins' constant. Sample B (N_w = 50.5) has almost identical properties. It was also studied by light scattering. The measured second virial coefficient was found to be positive and slightly lower than the magnitude expected on the basis of polymer studies. Overall mutual repulsion of chains, as observed in most light scattering studies of polymeric HA, is apparent. The repulsion, not apparent in the data for smaller fragments, is attributed to the existence of intramolecular associations, formed by chain folding. Similar structures, known as "hairpins," are adopted by DNA fragments of appropriate sequence and length (35, 36). The HA hairpin structures likely coexist with aggregates of the type adopted by shorter fragments, particularly at high sample concentrations. Sample C (N_w = 37.5) presumably exhibits a low magnitude second virial coefficient for this reason. The characteristics of both larger and smaller chains are particularly evident in this borderline size.

The proposed dimeric motif for interaction is supported by other previous studies of HA properties. The competitive inhibition of polymer-polymer interactions by addition of HA fragments, first noted by Welsh *et al.* (3), demands a basically dimeric model. X-ray fiber diffraction studies (4) show the feasibility, if not the general adoption, of double helical structures.

In a few previous reports, apparent aggregation of *polymeric* HA in neutral aqueous solution has been observed. Sheehan *et al.* (12) found a Na^+-dependent increase in the molecular weight measured by extrapolation of light scattering data to zero concentration. Silver and Swann (10) observed a similar phenomenon, as well as a negative apparent second virial coefficient, but only for a polymeric HA sample of somewhat reduced molecular weight. Our results for HA segments, however, are not directly comparable with these studies. We found no change in limiting molecular weight for any sample. We also found that chains of more than 35 disaccharides exhibit the more commonly observed positive second virial coefficient. It is possible that some of the observations for polymeric HA relate to sample handling procedures. These may strongly affect the relative degree of intra- versus intermolecular association. That is, nonequilibrium states may be adopted as a result of sample handling, or even preexist as a result of the biosynthetic process. For this reason, studies on polymeric HA have been subject to considerable variability among laboratories.

The self-association of HA segments in 0.15 M NaCl is apparently less strong than the self-association of dermatan sulfate chains in the same solvent (37, 38). For the latter case, extrapolation of low shear viscosity data or conventional static light scattering results to zero concentration results in the observation of high molecular weight aggregates. In KCl, dermatan sulfate chains exhibit a weaker interaction, similar to that observed here for HA in NaCl solution.

In summary, a weak self-association of HA segments can be demonstrated under physiological ionic conditions. Further studies on the thermodynamics and mechanism of association should be facilitated by using HA segments rather than polymeric HA samples.

ACKNOWLEDGMENTS

The authors thank Drs. John E. Gregory, W. Curtis Johnson, Jr., and Kenneth S. Schmitz for helpful discussions. This research was supported by National Institutes of Health Grant EY 04804. Preliminary accounts of this work were presented at the 31st Annual Meeting of the Biophysical Society, February 22-26, 1987, New Orleans, Louisiana (39), and at the Annual Meeting of the Association for Research in Vision and Ophthalmology, May 4-8, 1987, Sarasota, Florida (40).

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