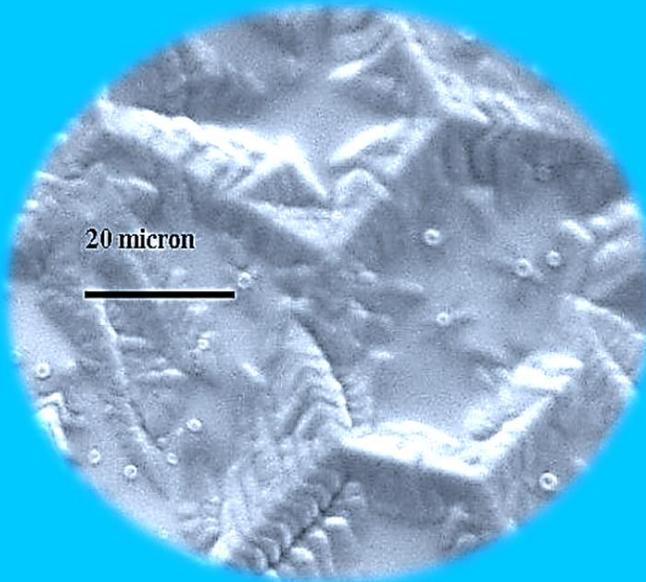


Hyaluronic Acid

Physical Properties



Raymond E. Turner
NYU Tandon (Formerly Brooklyn Poly)
1986

HYALURONIC ACID:
MOLECULAR WEIGHT
DEPENDENCY OF PHYSICAL
PROPERTIES

**HYALURONIC ACID: MOLECULAR WEIGHT DEPENDENCE OF
PHYSICAL PROPERTIES**

by *TURNER, RAYMOND EDWARD*

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Vita

The author was born in Portsmouth, Virginia on December 13, 1948 to Vernon and Kate Turner. He received his Bachelor of Science from Brooklyn College in 1974. After working several years as a research technician at Cornell University Medical College and Columbia University and upon completing a Master of Science degree from Fordham University, the author came to Polytechnic University in 1981 to pursue a doctoral degree in chemistry. He began his research in the laboratory of Mary K. Cowman in June of 1982. Dr. Cowman served as his research and dissertation advisor throughout his career at Polytechnic.

Dedication

I dedicate this dissertation to my beloved wife Margaret E. Turner, who gave me unfailing support during my final year at Polytechnic University. Additionally, I dedicate this work to my parents the late Vernon Turner Sr., and Kate E. Turner. Moreover, to both the late Melvina James and my godmother, Thersel Mack, for treating me like a son when I was a struggling student without family in New York. Thanks to my charming daughter, Ebony E. Turner, who has given me a reason to continue my struggle toward excellence and to Professor Mary

Cowman, who has given me the distinction of being her first graduate student.

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An Abstract

Sodium Hyaluronate: The Molecular Weight Dependency of Physical Properties

By

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Sodium hyaluronate, a major tissue glycosaminoglycan, when digested with specific enzymes produces a mixture of oligosaccharides having different degrees of polymerization. Cationic dyes, when used to stain enzymatically digested hyaluronic acid on polyacrylamide gels, show chain length dependence in their ability to bind these oligomers.

Purified oligomers of hyaluronic acid were produced by digestion of the polymer with bovine testicular hyaluronidase (a hexosaminidase) which cleaves the β (1 \rightarrow 4) linkages of hyaluronic acid, producing samples having various molecular weight distributions. Column chromatography and polyacrylamide gel methods were used to characterize the

segments. Dilute solution viscometry methods were used to study oligomers and oligomers plus polymer in order to determine if chain-chain association in hyaluronic acid had a minimum chain length requirement.

Circular dichroism was used to search for any spectral changes, which might be related to the extent of intermolecular association as a function of chain length. Of the different sizes of oligomers studied, some segments show evidence of intermolecular association in 0.15 M NaCl, but this only occurs in the presence of added polymer.

In perturbing solvent (0.15 M NaCl, pH 2.5, 17% ethanol), not only was a molecular weight

dependence found, but a concentration dependence of the CD spectra was also noted.

The results indicate that cationic dye binding to hyaluronic acid is chain length dependent and this dependency is due to a transition from non-interacting simple electrolyte behavior to interacting polyelectrolyte behavior. The range where maximal dye binding is obtained is dye type dependent. Hyaluronic acid is capable of forming intermolecular associations, which appear to be concentration dependent as well as chain length dependent. Dimer or higher aggregation occurs for hyaluronic acid with a weight-average number of disaccharides of 33 (HA33) in salt/ethanol/acid solvent. No evidence of aggregation was found in 0.15M NaCl for

segments alone. In some cases, a matrix or the presence of polymer may be required before association can occur. A dynamic molecular weight dependent model for hyaluronic acid association is proposed.

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Chapter 1: Introduction

Hyaluronic Acid Distribution in Animal Tissue

Hyaluronic acid (abbreviated HA) is a high molecular weight linear charged polymer, which is a major component of most connective tissue. Hyaluronic acid belongs to a class of compounds known as mucopolysaccharides or glycosaminoglycans. The molecule is composed of alternating N-acetyl- β -D-glucosamine and β -D-glucuronic acid residues linked at positions 1, 3 and 1, 4 respectively. Meyer and Palmer discovered hyaluronic acid in bovine vitreous in

1934.¹ The authors used the acetone precipitate of fresh cattle vitreous humor as starting material. The acetone powder from 100 eyes afforded a yield of 0.73 grams. The powder was extracted several times with acetic acid, washed with ethanol, suspended in alkaline solution and centrifuged. The supernatant was precipitated and washed several times with acetone, ethanol, and ether and dried in vacuo over phosphorus pentoxide. The authors suggested the name “hyaluronate” because the substance was

¹ Meyer, Karl, and John W. Palmer. "The polysaccharide of the vitreous humor." *Journal of Biological Chemistry* 107, no. 3 (1934): 629-634.

obtained from hyaloid (vitreous) and contained uronic acid.

The umbilical cord preparation yielded a sulfate free material resembling that found in vitreous and which contained glucosamine. It was found in conjunction with a material, which was identified as chondroitin sulfate.

Glycosaminoglycans can be found in many different tissues. The presence of “mucin” in skin, a connective tissue, was discovered in 1958 by Rollet as described by Pearce.² The “spreading effect” as injected ink spread through

² Pearce, R. H. "Glycosaminoglycans and glycoproteins in skin." *The amino sugars* 2 (1965): 149-193.

skin membrane was found to be dependent on the hyaluronidase activity of the injected material. This suggests that the glycosaminoglycan component of the skin may be altered by the depolymerizing and hydrolyzing effect of the enzyme. Hyaluronic acid is the major glycosaminoglycan isoionic extracts of rabbit skin as well. The concentration of glycosaminoglycan in human skin is between 43 to 145 mg per 100-gram fresh weight (with variation due to age).

Paul Ehrlich, who used cationic dyes to stain basophilic cells of connective tissue, discovered “Metachromasia” in mast cells about 100 years ago. Although glycosaminoglycans are found in mast cells and in mast cell tumors,

hyaluronic acid does not appear to be present.³ Mast cells produce acid polysaccharides for the intercellular space of connective tissue.

Hyaluronic acid was found to be one of the main glycosaminoglycans in the umbilical cord.⁴ Hence, hyaluronic acid serves as a cushion protecting the umbilical cord from compression. The arterial wall contains large amounts of glycosaminoglycans, which can be stained with metachromatic dyes such as toluidine blue. Immature aorta shows metachromasia, which is

³ Ringertz, Nils R. "Glycosaminoglycans in mast cells and in mast-cell tumors." *The Amino Sugars 2* (1965): 209-217.

⁴ Szirmai, J. A. "Glycosaminoglycans in umbilical cord." *The Amino Sugars 2* (1965): 219-227.

destroyed by bacterial hyaluronidase. The amount of hyaluronic acid in arteries and blood vessels changes with age and is sometimes replaced by chondroitin sulfate. New connective tissue formed on homografts leads to formation of a new endothelial layer, which shows intense fibroblast activity. Also produced is metachromatic material, which is initially digested with hyaluronidase, but becomes increasingly more difficult to digest with age.⁵

Morphologically, proteoglycans exist in extracellular matrices of hyaline cartilage as

⁵ Muir, Helen. "Glycosaminoglycans and glycoprotein in blood-vessel walls." *The amino sugars* 2 (1965): 311.

large aggregates where many monomers are bound to a single large molecule of hyaluronic acid.⁶ The HA binding region has been identified as located at one end of the proteoglycans. The interaction of the HA binding region with HA is highly specific for HA.

Hyaluronic Acid in Vitreous and Joint

The concentration of hyaluronic acid in both the liquid gel and liquid vitreous of the human eye is very low at birth, but increases with age

⁶ Kimura, James H., Timothy E. Hardingham, Vincent C. Hascall, and Michael Solursh. "Biosynthesis of proteoglycans and their assembly into aggregates in cultures of chondrocytes from the Swarm rat chondrosarcoma." *Journal of Biological Chemistry* 254, no. 8 (1979): 2600-2609.

until around age 20 after which case the levels remain constant throughout adulthood. Hyaluronic acid concentration in gel has so far been found to be greater than that of the liquid vitreous.^{7 8} In addition, a study of the synovial fluids of human males both young and old has shown that the concentration and the chain length (molecular size) of hyaluronic acid remained invariant with age. However, changes in the viscoelastic properties and the ability to

⁷ Balazs, E.A., and Denlinger, J.L. *Fondazione Ponci* 34 (6) (1979) 637-647.

⁸ Balazs, Endre A., Torvard C. Laurent, and Ulla BG Laurent. "Studies on the structure of the vitreous body VI. Biochemical changes during development." *Journal of Biological Chemistry* 234, no. 2 (1959): 422-430.

store mechanical energy in the form of elasticity was reported to change with age.⁷

Balazs and others studied the vitreous body of different animal species and found that of the animals studied, only the squid and the owl monkey contained entirely liquid vitreous.⁹ In hens, turkeys, and pigeons the portion next to the retina is liquid while the vitreous body behind the lens is solid gel. The turnover rate of hyaluronic acid has been shown to be higher for synovial fluid than vitreous.⁷ For owl monkeys

⁹ Balazs, Endre A., Torvard C. Laurent, Ulla BG Laurent, Marcella H. DeRoche, and Doris M. Bunney. "Studies on the structure of the vitreous body: VIII. Comparative biochemistry." *Archives of biochemistry and biophysics* 81, no. 2 (1959): 464-479.

and rhesus monkeys it is about 3 to 4 months compared to only 5 to 7 days for rabbit and horse joints. Ogston and Stanier¹⁰ observed that the viscosity of synovial fluid varies with shear stress and suggested that the shear elasticity of synovial fluid might be an important aspect of its function in joints.

Water soluble glycosaminoglycans of connective tissue affect the bulk flow of water and the diffusion of solute in model systems as well as in tissue.¹¹ Preston et al ¹¹ ¹² studied the

¹⁰ Ogston, A. G., J. E. Stanier, B. A. Toms, and D. J. Strawbridge. "Elastic properties of ox synovial fluid." *Nature* 165, no. 4197 (1950): 571-571.

¹¹ Preston, B. N., and J. McK Snowden. "Model

diffusional behavior of low molecular weight solute through gel membranes containing polyanionic material used as a model for connective tissue. They found that solution of "native" proteoglycans with a gel-agarose had little or no effect on the diffusion of non-electrolytes, but markedly affected the behavior of ionic species. Counter-ion fixation occurs.

Laurent et al¹³ studied the diffusion of

connective tissue system: The effect of proteoglycan on the diffusional behavior of small non-electrolytes and microions." *Biopolymers* 11, no. 8 (1972): 1627-1643.

¹² Preston, B. N., and J. McK Snowden. "Model connective tissue system: The effect of proteoglycan on the diffusional behavior of small non-electrolytes and microions." *Biopolymers* 11, no. 8 (1972): 1645-1659.

¹³ Laurent, Torvard C., Ingemar Björk, Adolph

albumin, alpha-crystallin, fibrinogen, and turnip yellow mosaic virus in HA media. They found a decreased rate for the substances in HA media relative to pure solvent. The relative decrease of the sedimentation rate of a particle was ascribed to the molecular sieving effect of HA. Chain entanglement of the extended coil structure of HA can occur at concentrations as low as 1 mg/ml and can lead to a three dimensional network structure and thus act as a molecular sieve.

Pietruszkiewicz, and Håkan Persson. "On the interaction between polysaccharides and other macromolecules: II. The transport of globular particles through hyaluronic acid solutions." *Biochimica et biophysica acta* 78, no. 2 (1963): 351-359.

Balazs and others¹⁴ studied this chemical and morphological fine structure of the cartilage surfaces and joints. It was suspected at this time that the relationship between glycosaminoglycans and collagen fibers must be understood in order to account for the lubrication process known to occur in joint tissue. The outermost surface of the articular cartilage is an adsorbed hyaluronic acid layer and the underlying cartilage still considered to be superficial, contains hyaluronic acid as well as some keratan sulfate.

¹⁴ Ea, Balazs, G. D. Bloom, and D. A. Swann. "Fine structures and glycosaminoglycan content of the surface layer of articular cartilage." In *Fed Proc*, vol. 25, pp. 1813-1816. 1966.

Hyaluronic Acid in Cartilage and Proteoglycan Aggregation

Hyaluronic acid can interact with proteoglycans to form large feather-like aggregates. It was suggested that a large number of proteoglycans were bound to HA chains and that each proteoglycan contained only a single binding site.¹⁵ ¹⁶It was further suggested a glycoprotein link is essential for this aggregation

¹⁵ Hardingham, Timothy E., and Helen Muir. "Binding of oligosaccharides of hyaluronic acid to proteoglycans." *Biochemical Journal* 135, no. 4 (1973): 905-908.

¹⁶ Hardingham, Timothy E., and Helen Muir. "The specific interaction of hyaluronic acid with cartilage proteoglycans." *Biochimica et Biophysica Acta (BBA)-General Subjects* 279, no. 2 (1972): 401-405.

of proteoglycan subunits.¹⁷

Hascall and Sajdera^{15 18} used two methods to isolate proteoglycan subunits from bovine nasal cartilage. In the first method, the protein-polysaccharide complex in 0.5 M guanidinium chloride, 0.5 M sodium acetate, pH 5.8 was diluted with an equal volume of 7.5 M GnCl and CaCl per gram solution. A density gradient was formed by centrifugation at 4000 rpm for 40 hours at 20 °C in a Spinco S.W. 50 rotor.

¹⁷ Hascall, Vincent C., and Stanley W. Sajdera. "Protein-polysaccharide complex from bovine nasal cartilage The function of glycoprotein in the formation of aggregates." *Journal of Biological Chemistry* 244, no. 9 (1969): 2384-2396.

¹⁸ Hascall, Vincent C., and Stanley W. Sajdera. "Physical properties and polydispersity of proteoglycan from bovine nasal cartilage." *Journal of Biological Chemistry* 245, no. 19 (1970): 4920-4930.

Fractions were isolated, their densities determined and dialyzed against 0.05 M GnCl , pH 8.5 buffer. The proteoglycan subunit was isolated from the bottom 2/5 of the tubes and glycoprotein linked fractions -from the top 1/5.

The second method was a solidification of the first method and allowed only the proteoglycan to be isolated from the bottom 2/5 of the tube. Once separated, this protein-polysaccharide complex did not reassociate in associative solvent unless combined with the glycoprotein fraction, It was found that the aggregates in protein- polysaccharide complexes are composed of an undetermined number of proteoglycan subunit molecules. The glycoprotein linked fraction is essential for aggregation of this

proteoglycan subunit. Reduction of disulfide bonds or denaturing the protein with denaturing solvents prevents aggregation. Proteoglycan contains a single class of macromolecules having a mean molecular weight of 2.5 million and a standard deviation of 1.2 million. It was also found that proteoglycan subunits contain only one type of protein and that polydispersity is the result of varying amounts of chondroitin sulfate and Keratin sulfate attached to protein.

Hardingham and Muir¹⁹ found that the addition of small amounts of HA to

¹⁹ Hardingham, Timothy E., and Helen Muir. "Hyaluronic acid in cartilage and proteoglycan aggregation." *Biochemical Journal* 139, no. 3 (1974): 565-581.

disaggregated cartilage proteoglycan produces a large increase in the hydrodynamic size as determined by gel chromatography.¹⁵The interaction was accompanied by a large increase in viscosity which was eliminated in concentrated solutions of guanidine HCl at low pH. It was estimated that 10-30 proteoglycans were associated with each hyaluronic acid chain.

Rheological Studies of Hyaluronic Acid in Vitreous and Synovial Fluid

Vitreous humor consists of connective tissue space made up of intercellular matrix components. The two major elements of these

matrices are collagen and the hyaluronic acid networks.²⁰ The collagen fibers are not cross-linked whereas the hyaluronate network appears to be extensively cross-linked. The nature of this cross-linking is a subject of debate. The plasticity of vitreous is attributed to the presence of collagen while the elasticity is attributed to the presence of hyaluronic acid. Balazs²⁰ uses a biological model and correlates four fundamental physiologic properties of the vitreous with four physiochemical principles.

²⁰ Balazs, E. A. "The molecular biology of the vitreous." In *New and Controversial Aspects of Retinal Detachment*, pp. 3-15. Harper & Row, New York, 1968.

They are (1) the frictional interaction between the hyaluronic acid network and the collagen fibers, (2) the vitreous expansion and contraction, (3) the excluded volume effect, and (4) the molecular sieve effect.

Hyaluronic acid provides support for the vitreous and prevents the vitreous from collapsing when subjected to mechanical agitation and also prevents phase separation in vitreous. There, hyaluronic acid can stabilize the collagen fibers through frictional interaction. This stabilization effect was found to be dependent on hyaluronic acid concentration and molecular weight. Due to the presence of charged species in vitreous such as protein and counter-ions, hyaluronate experiences a

restricted volume.

Hyaluronic acid can therefore contract or expand depending on the nature of the effective charge on the polymer. Washing the vitreous causes the removal of sodium counter-ions and chloride by ions causing the gel vitreous to swell due to the mutual repulsion of the HA molecules.²⁰ Space already occupied by the collagen fibers can exclude the HA molecule. Together HA and collagen exclude non-collagen protein and other macromolecules from vitreous.

Balazs et al⁷ gives us additional insight into the role of HA by comparing the vitreous morphology with that of joint. An ideal joint is visualized and compared with the ideal vitreous. In both models there is a liquid compartment

filled with a viscous fluid. This pocket is surrounded by a loose collagen network which forms a gel differing in rigidity. The central liquid pocket is synovial fluid in joints and is called the liquid vitreous in vitreous humor. In joint, a loose collagenous tissue surrounds the synovial fluid and an additional interface occurs at the articular cartilage at the base of the bone, which makes contact with the fluid.

Similarity between the two compartments exist in hyaluronic acid content. Synovial fluid and liquid vitreous are the only two compartments in the body which are vitreous because they contain high polymeric HA. In addition HA is the only glycosaminoglycan present.

Much insight into the physiological role of HA in vitreous and in synovial fluid can be obtained by studying HA in solution. Dintenfass²¹ contends that both synovial fluid and the articular cartilage play an important role in joint lubrication. The author studied samples of synovial fluid obtained from the knees of individual patients using a cone in cone viscometer. Fluids obtained from normal persons and traumatic arthritis cases were always thixotropic while the fluid obtained from rheumatoid arthritis cases was found to be

²¹ Dintenfass, L. "Rheology of complex fluids and some observations on joint lubrication." In *Federation proceedings*, vol. 25, no. 3, pp. 1054-1060. 1965.

newtonian. Newtonian systems have viscosities independent of time and rate of shear (viscosity relates shear stress and rate of shear). While thixotropic systems have viscosities dependent on time and rate of shear. The viscosity decreases with increasing rate of shear up to some crucial rate of shear above which a plateau region is attained.

Viscosity and thixotropy of synovial fluid are due to their hyaluronic acid content. It was shown (21 and the references therein) that a breakdown of polysaccharide with hyaluronidase or the removal of HA by acetic acid precipitation reduces the viscosity of synovial fluid to that of water. It appears that the rheological changes in synovial fluid might be due to molecular

aggregation, intermolecular adhesion, and binding.

Gibbs et al²² studied the viscoelasticity of hyaluronic acid over a frequency range of 0.02 to 1.67 cps. The effect of varying temperature, pH, concentration, and ionic strength on the dynamic shear module was studied. The author found a maximum elastic effect at pH 2.5, which was not observed at lower or higher pH. No evidence of entanglement coupling of HA molecules was found within the concentration range studied (2.0 to 4.0 mg/ml). An oscillating

²² Gibbs, David A., E. W. Merrill, K. Af Smith, and E. A. Balazs. "Rheology of hyaluronic acid." *Biopolymers* 6, no. 6 (1968): 777-791.

couette rheometer was used to measure the dynamic viscoelastic properties of HA solutions. These measurements made under the different conditions were expressed in terms of two reduced variables.

The elastic modulus (G') and the storage modulus (G''), which was plotted as a function of frequency. Two regions in the curves might be distinguished. Below the G' and G'' intersection (the internal zones) G'' , the storage modulus, predominates and the elasticity falls below zero. Within the time scale of the experiment, stress relaxation is attained and the sample appears as a viscous liquid. Above the point where the two curves intersect, the solution appears elastic because the elastic modulus predominates. A

plateau region is reached known as the elastic region.

The master curves obtained at three different pHs were very similar in shape. It was concluded that only relaxation rates and not the nature of the relaxation is important in HA polymer. Therefore the introduction of covalent intermolecular cross-links was ruled out. When compared to other polymers the G' and G'' curves are unique. The transition region between the terminal and the elastic regions is virtually non-existent.

This suggests that the transition from a relative rigid elastic system to a viscous fluid does not involve extensive relaxation and uncoupling of the glycosidic chains of the HA

molecule. Although the concentration range used in this study was more than enough for coil overlap. No entanglement coupling was found, but rather a relaxation mechanism involving the breakdown of a highly elastic hydrogen bonded network followed by viscous flow was proposed.

Tissue Isolation of hyaluronic acid

Hyaluronate can be isolated from tissue by a variety of methods. The simplest method involves the use of water or salt solution. Once in solution, hyaluronate can be precipitated from the solution by either organic solvent or

quaternary ammonium salts such as cetylpyridinium chloride.²³ –The precipitates are soluble in salt solutions at a critical electrolyte concentration. For a given organic precipitant, polyanionic mixtures are fractionated according to their various CECs in a given salt solution. One problem is that not all the protein is removed.

Hyaluronate has a very high molecular weight and an even greater molecular volume when compared to protein of synovial fluid. This allows

²³ Laurent, T. C, and J. E. Scott. "Molecular weight fractionation of polyanions by cetylpyridinium chloride in salt solutions." *Nature* 202 (1964): 661-662.

for separation of HA on special filters, which allow proteins to pass, but not hyaluronate.²⁴

Hyaluronate oligosaccharides can occur naturally as a product of catabolism or can be obtained through chemical or enzymatic degradation.²⁵ Oligosaccharides can be identified by paper chromatography. As a rule, the general methods and solvent systems employed in carbohydrate chemistry apply for nitrogen containing oligosaccharides. Such solvent mixtures as ethyl acetate-pyridine-water

²⁴ Hammerman, David, and John Sandson. "Isolation of hyaluronate from human synovial fluid by zone electrophoresis." *Nature* 188, no. 4757 (1960): 1194-1195.

²⁵ Balazs, E.A, and Jeanloz, R.W. *The Amino Sugars* Vol. 1a Academic Press (1969).

and butanol-ethanol-water have been employed.

Oligosaccharides absorption occurs on charcoal celite columns and on powdered cellulose.²⁵ Barker²⁶ and Rupley reported the separation of acetamido oligosaccharides derived from chitin.²⁷

Shimada and Matsumura²⁸ precipitated crude

²⁶ Barker, S. A., A. B. Foster, M. Stacey, and J. M. Webber. "451. Amino-sugars and related compounds. Part IV. Isolation and properties of oligosaccharides obtained by controlled fragmentation of chitin." *Journal of the Chemical Society (Resumed)* (1958): 2218-2227.

²⁷ Rupley, J. A. "The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular-weight substrate for lysozyme." *Biochimica et Biophysica Acta (BBA)-Specialized Section on Mucoproteins and Mucopolysaccharides* 83, no. 3 (1964): 245-255.

²⁸ Shimada, E, and G. Matsumura. "Viscosity and molecular weight of hyaluronic acids." *The Journal of*

glycosaminoglycans with cetylpyridinium chloride from 0.5 M NaCl extract from dry defatted rabbit skin powder followed by successive extractions with 0.34, 1.2, and 2.1 M NaCl and 0.5 M NaOH. Three volumes of ethanol were then used to recover the material. DEAE Sephadex A-25 Cl form was used to elute fractions in 0.5M NaCl. A step gradient of salt was used to isolate different molecular weight fractions.

Laurent et al²⁹ fractionated HA from bovine

Biochemistry 78, no. 3 (1975): 513-517.

²⁹ Laurent, Torvard C., Marion Ryan, and Adolph Pietruszkiewicz. "Fractionation of hyaluronic acid the polydispersity of hyaluronic acid from the bovine

vitreous bodies based on their solubility in Na_4SO_4 solution. An 89% yield with a protein contamination of only 2% was obtained.

Cleland³⁰ purified the crude potassium salt of hyaluronate obtained from bovine vitreous humor. The sample was dissolved in 500 ml water containing 1.25 mg of 5, 7 dichloro-8-quinolinol (DQC) as preservative, centrifuged for 15 minutes at 9000 x g, and the supernatant dialyzed against water for three days. The

vitreous body." *Biochimica et biophysica acta* 42 (1960): 476-485.

³⁰ Cleland, Robert L. "Ionic polysaccharides. II. Comparison of polyelectrolyte behavior of hyaluronate with that of carboxymethyl cellulose." *Biopolymers* 6, no. 11 (1968): 1519-1529.

dialysate was treated with kaolin and re-centrifuged as above. The hyaluronate was converted to the acid form with Dowex 50W-X8 in the H⁺ form.

Structure of HA in Solution and in the Solid State

Unlike the case for proteins, which are linearly linked amino acids; polysaccharides are joined in a much more complex way. The primary structure of a polysaccharide would include the type of monosaccharides present, sequence of these residues, position and anomeric configuration of linkages, and the position of other constituents. Despite the rigidity of the individual sugar residues, rotation about the glycosidic linkages is possible and

determines the overall conformation of the polysaccharide. Hyaluronic acid is a linear polymer and four torsional angles need to be specified to determine geometry.³¹

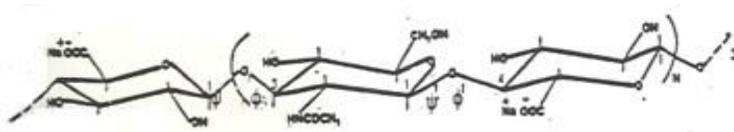


Figure 1. Sodium hyaluronate, MW: 799.6366. Taken from ChemIDplus A TOXNET DATABASE, NIH, US National Library of Medicine.

The value of ψ , ψ' and Φ and Φ' are restricted by steric hindrance between the adjacent rings and by non-bonded interactions between groups in adjacent residues. The most stable form is the

³¹ Kennedy, John F., and Charles A. White. *Bioactive carbohydrates: in chemistry, biochemistry and biology*. Ellis Horwood Ltd., 1983.

4C_1 chair form with all the ring substituents in the equatorial position. The primary structure for the most part determines the secondary and tertiary structure. Temperature, ionic strength, and the nature of the counter-ion also affect the secondary structure and the tertiary structure. Non-covalent association of subunits or aggregation describes higher order structure.

Tritium exchange methods in aqueous solution show exchange rates for the acetamido portion relative to model compounds. Participation of the acetamido hydrogen in a labile intermolecular hydrogen bond was

suggested.³² This idea gains support from the results of Scott et al³³ who reported a reduced cleavage rate for C₂-C₃ OH groups of the glucuronic acid when the polymer was subjected to periodic acid oxidation. Also Scott et al,³⁴ working in DMSO, concluded from proton NMR data that intermolecular hydrogen bonding

³² Oberholtzer, J. C., S. W. Englander, and A. F. Horwitz. "Hydrogen-bonded conformation of hyaluronate oligosaccharide fragments in aqueous solution." *FEBS Letters* 158, no. 2 (1983): 305-309.

³³ Scott, John E., and MICHAEL J. Tigwell. "Periodate oxidation and the shapes of glycosaminoglycuronans in solution." *Biochemical Journal* 173, no. 1 (1978): 103-114.

³⁴ Scott, John E., F. Heatley, D. Moorcroft, and A. H. Olavesen. "Secondary structures of hyaluronate and chondroitin sulphates. A ¹H NMR study of NH signals in dimethyl-sulphoxide solution." *Biochemical Journal* 199, no. 3 (1981): 829-832.

occurs in HA. However, ¹H NMR work by Cowman et al³⁵ indicates that hydrogen bonding to solvent water might predominate for hyaluronic acid in aqueous solution.

Silver and Swann³⁶ found that the second virial coefficient, which depends on the magnitude of the intermolecular forces in the polymer, decreases with corresponding molecular weight for hyaluronic acid salt solution.

³⁵ Cowman, Mary K., Dennis Cozart, Koji Nakanishi, and Endre A. Balazs. "1H NMR of glycosaminoglycans and hyaluronic acid oligosaccharides in aqueous solution: the amide proton environment." *Archives of biochemistry and biophysics* 230, no. 1 (1984): 203-212.

³⁶ Silver, Frederick H., and David A. Swann. "Laser light scattering measurements on vitreous and rooster comb hyaluronic acids." *International Journal of Biological Macromolecules* 4, no. 7 (1982): 425-429.

The persistence length of HA in 0.05M nitric acid was found to be between 4 nm and 6 nm determined from small angle x-ray scattering measurement.³⁷ In 0.2 M NaCl, the persistence length, $a=4$ nm while the data for $[\eta]$ vs. M_w gave the results expected for a worm-like chain model of persistence length =4 nm.

For a low molecular weight preparation of HA of 2.7×10^4 , the radius of gyration was found to be 8 nm. Laurent et al³⁸ determined from light

³⁷ Cleland, Robert L. "The persistence length of hyaluronic acid: an estimate from small-angle X-ray scattering and intrinsic viscosity." *Archives of biochemistry and biophysics* 180, no. 1 (1977): 57-68.

³⁸ Laurent, T. C., and J. Gergely. "Light scattering studies on hyaluronic acid." *Journal of Biological Chemistry* 212, no. 1 (1955): 325-333.

scattering on umbilical cord HA having a weight average molecular weight between 2.8×10^6 and 4.3×10^6 , a polymer radius of gyration of between 180 -240 nm, which is in agreement with a random coil model.

Shimada and Matsumura²⁸ examined the double log plot of $[\eta]$ vs. Mw for polymers having different degrees of polymerization. These data yield values of a K for the Mark-Houwink relationship. For low molecular weight fractions less than 2.4×10^4 , the relationship $[\eta] = 3.0 \times 10^{-4} M^{1.2}$ was applicable. High molecular weight polymers greater than 1.5×10^5 gave the

relationship $[\eta] = 5.7 \times 10^{-2} M^{0.76}$. Cleland and Wang³⁹ reported values of a and K of 0.816 and 0.0228 in 0.2 M NaCl, and 0.777 and 0.0318 in 0.5 M NaCl, respectively.

In addition, Cleland⁴⁰ found that for small sizes of approximately 22×10^3 $[\eta] / M_w = 0.0028$. This compares to a value of 0.0020 found by Shimada and Matsumura.²⁸ X-ray diffraction studies can yield valuable information concerning the secondary structure of

³⁹ Cleland, Robert L., and John L. Wang. "Ionic polysaccharides. III. Dilute solution properties of hyaluronic acid fractions." *Biopolymers* 9, no. 7 (1970): 799-810.

⁴⁰ Cleland, Robert L. "Viscometry and sedimentation equilibrium of partially hydrolyzed hyaluronate: comparison with theoretical models of wormlike chains." *Biopolymers* 23, no. 4 (1984): 647-666.

polysaccharide chains and the mode of cationic dye binding in glycosaminoglycans. Hyaluronic acid, chondroitin-4-sulfate, and chondroitin-6-sulfate can adopt the 3-fold and 2-fold helix while chondroitin-6-sulfate and dermatan sulfate can both adopt the 8-fold conformation.⁴⁰

In solution, hyaluronic acid conformation is counter-ion dependent. In the presence of the divalent ion, Ca^{2+} , and at 75 to 92% relative humidity stretched films, the 3_2 helix is preferred. This is attributed to cooperative Ca^{2+} binding and further aggregation.⁴¹ Sheehan et

⁴¹ Winter, W. T., and Struther Arnott. "Hyaluronic acid: the role of divalent cations in conformation and packing." *Journal of molecular biology* 117, no. 3

al⁴² stress the importance of the counter-ion to the prevailing helical structure. The diffusion in tissue of certain ion types might provide the driving force for the interconversion of different helical structures.

Films of HA under conditions in which the polymer is partially mixed with potassium counter-ions form left-handed ant parallel double helical structures. In solution, the rapid transformation from liquid to solid character with increasing shear stress might be related to

(1977): 761-784.

⁴² Sheehan, J. K., K. H. Gardner, and E. D. T. Atkins. "Hyaluronic acid: a double-helical structure in the presence of potassium at low pH and found also with the cations ammonium, rubidium and cesium." *Journal of molecular biology* 117, no. 1 (1977): 113-135.

double helix cross links.⁴³ HA exists as a fully extended 3-fold helix in the presence of sodium ions. HA also exist in the less extended 4-fold helical form. ⁴⁴ Stiff chain segments could result from double helical loops, which could account for the characteristic viscoelastic properties of HA solution.⁴⁵

⁴³ Dea, I. C. M., R. Moorhouse, D. A. Rees, S. Arnott, J. M. Guss, and E. A. Balazs. "Hyaluronic acid: a novel, double helical molecule." *Science* 179, no. 4073 (1973): 560-562.

⁴⁴ Winter, W. T., P. J. C. Smith, and Struther Arnott. "Hyaluronic acid: structure of a fully extended 3-fold helical sodium salt and comparison with the less extended 4-fold helical forms." *Journal of molecular biology* 99, no. 2 (1975): 219-235.

⁴⁵ Mitra, A. K., Struther Arnott, E. D. T. Atkins, and D. H. Isaac. "Dermatan sulfate: molecular conformations and interactions in the condensed state." *Journal of molecular biology* 169, no. 4 (1983): 873-901.

Cationic Dye Binding to Hyaluronic Acid: Early Studies

Metachromasy of dyes in solution is well documented. In a review by Paddy⁴⁶ metachromasy is described as follows:

“Metachromasy is a reversible color change that any dye may undergo by virtue of a change in its environment not involving chemical reaction of the dye.”

A distinction is made between the unperturbed dye and the perturbed dye. The unperturbed state of the dye is taken as that characterized by its spectrum in dilute solution of n-alkanoids or

⁴⁶ Paddy, J.F in Chemistry and Molecular Biology of the Intracellular Matrix (Balazs, E.A., ed.) vol. 2, Academic Press, NY (1970): 1007-1031

acetone. Some of the main features responsible for metachromasia in solution are (1) concentration of dye, (2) salt concentration, (3) temperature, (4) dissociation, (5) nature of the solvent, (6) dye structure, (7) and the polyion of the opposite charge to the dye.

In dilute solution, the spectra of many dyes have the same shape as those in alcoholic solution and show an extremely concentration dependent new peak. The peaks are described as “M” for the main peak resembling the unperturbed state and “D” for the new peak appearing as a shoulder on the main peak thought to be due to the dimeric state of the dyes. Higher aggregation of the dye is attributed

to a peak called the “H” band. All spectra are referred to the unperturbed state.

Work done by Paddy^{47 48} on the effects of counter-ions on metachromasy indicates that the counter-ion was not involved in the observed metachromatic effect and that the evidence strongly suggests aggregation. However, a small effect was in fact noticed. Salt usually favors aggregation of dye (dimer formation). High salt reduces the electrostatic field around the dimer and its’ opposite charge and alters the free energy of dimer formation. Divalent ions are

⁴⁷ Paddy, J.F., J. Phys. Chem. (1967) 71, 3488-3493.

⁴⁸ Paddy, J.F., J. Phys. Chem. (1968) 72, 1259-1264.

therefore more effective than monovalent ions. X-ray evidence indicates that the aggregation of cyanine dyes is arranged as though the dye ions were a stack of cards (Trans and face to face).

Water-water interaction is a strong driving force for aggregation. Thus, the term “hydrophobic bonding” has been introduced to explain this effect, which leads to an increase in entropy for the system. Color change brought about by two or more dye chromophores being forced together is due to exciton coupling.

More relevant to the work presented here are the spectral changes observed for cationic dyes due to interaction with macromolecules.

Kay et al⁴⁹ studied the influence of pH, temperature and dye-macromolecule ratios on the spectral changes induced by macromolecules in the dye 4, 5, 4', 5'-dibenzo-3, 3'-diethyl-9-methylthiacarbocyanine bromide. Their results indicate that the observed spectral changes are due to the adsorption of the dye onto the macromolecule and the adsorption process depends upon electrostatic forces and that the nature of the macromolecule is the most important factor in determining the changes in

⁴⁹ Kay, Robert E., E. Richard Walwick, and Cheryl K. Gifford. "Spectral Changes in a Cationic Dye Due to Interaction with Macromolecules. I. Behavior of Dye Alone in Solution and the Effect of Added Macromolecules1." *The Journal of Physical Chemistry* 68, no. 7 (1964): 1896-1906.

the observed dye polymerization.

Dye binding to DNA is interesting in that aggregation does not occur when the molecule is in the native state, but occurs upon denaturation. Apparently, in the denatured state, dye-dye interaction is more favorable and this alters the energy levels of the excited states of the dye causing a hypsochromic shift typical of most aggregated states.

Stone and Bradley⁵⁰ studied the aggregation of cationic dyes on acidic polysaccharides using

⁵⁰ Stone, Audrey L., and Dan F. Bradley. "Aggregation of cationic dyes on acid polysaccharides I. Spectrophotometric titration with acridine orange and other metachromatic dyes." *Biochimica et Biophysica Acta (BBA)-General Subjects* 148, no. 1 (1967): 172-192.

spectrophotometric titration. The authors used four metachromatic dyes. However, acridine orange was applicable for all classes of polymers studied. It was found that in general the dyes form 1:1 (site: dye) complexes with most of the cationic polysaccharides.

When hyaluronic acid or sulfated polysaccharides are mixed with cationic dyes in aqueous solution at room temperature, one could observe a monochromatic shift in the absence of precipitation, precipitation without a change in spectra, or both metachromasia and precipitation.⁵¹ As long as the number of anionic

⁵¹ Szirmai, J. A., and E. A. Balazs. "Metachromasia

sites of the poly-acid is well in excess of the number of dye molecules, the unsaturated complex is soluble and follows the law of Beer and Lambert. When the saturation point of the anionic site is reached, poly-acid dye complex precipitates. Removal of the precipitate leaves a poly-ion dye solution with two distinct types of curves. One represents the saturated anionic polymer and the other represents the excess of free dye.

Hyaluronic acid has one carboxyl group per disaccharide and does not bind azure A when

and the quantitative determination of dye binding." *Acta histochemica. Supplementband* (1958): 56.

the reaction mixture is below pH3.

Scott⁵² studied the critical electrolyte effect in the interaction between polyanions and organic cations. A large number of cationic dyes have been examined in the CEC system and show high affinity for the polynucleotides due to interaction between aromatic rings. Proteoglycans lack such aromatic rings and as a result show a lower affinity for dye binding. In searching for a dye, which would stain glycosaminoglycans, alcian blue was found to be

⁵²Scott, J. E. "Critical electrolyte concentration (CEC) effects in interactions between acid glycosaminoglycans and organic cations and polycations." *Chemistry and molecular biology of the intercellular matrix 2* (1970): 1105-1119.

a suitable candidate.

In the present study, we are concerned with the visualization of oligomers of hyaluronic acid using a 10% polyacrylamide gel electrophoresis method. Although oligosaccharides differing in chain length can be separated by gel chromatography,^{53 54} ion exchange methods,^{55 56}

⁵³ Cowman, Mary K., Endre A. Balazs, Carl W. Bergmann, and Karl Meyer. "Preparation and circular dichroism analysis of sodium hyaluronate oligosaccharides and chondroitin." *Biochemistry* 20, no. 5 (1981): 1379-1385.

⁵⁴ Shimada, E., and Matsumura, G., *J. Biochemistry* (1981)96, 721-725.

⁵⁵ HÖÖ, Magnus, Ulf Lindahl, and Per-Henrik Iverius. "Distribution of sulphate and iduronic acid residues in heparin and heparan sulphate." *Biochemical Journal* 137, no. 1 (1974): 33-43.

⁵⁶ Fransson, Lars-Åke. "Structure of dermatan sulfate III. The hybrid structure of dermatan sulfate from umbilical cord." *Journal of Biological Chemistry*

57 58 59 or by HPLC methods,^{60 61 62}the gel

243, no. 7 (1968): 1504-1510.

⁵⁷ Fransson, Lars-Åke, and Anders Malmström. "Structure of Pig Skin Dermatan Sulfate." *European Journal of Biochemistry* 18, no. 3 (1971): 422-430.

⁵⁸ Fransson, Lars-Åke. "Structure of dermatan sulfate: IV. Glycopeptides from the carbohydrate-protein linkage region of pig skin dermatan sulfate." *Biochimica et Biophysica Acta (BBA)-General Subjects* 156, no. 2 (1968): 311-316.

⁵⁹ Månsson, P., Ö. Jacobsson, and K. A. Granath. "Effect of the cations sodium and potassium on the molecular weight of hyaluronate." *International Journal of Biological Macromolecules* 7, no. 1 (1985): 30-32.

⁶⁰ Delaney, Stephen R., Marilyn Leger, and H. Edward Conrad. "Quantitation of the sulfated disaccharides of heparin by high performance liquid chromatography." *Analytical biochemistry* 106, no. 1 (1980): 253-261.

⁶¹ Delaney, Stephen R., H. Edward Conrad, and Janet H. Glaser. "A high-performance liquid chromatography approach for isolation and sequencing of chondroitin sulfate oligosaccharides." *Analytical biochemistry* 108, no. 1 (1980): 25-34.

⁶² Corbett, R. J., and Rodney S. Roche. "Use of high-speed size-exclusion chromatography for the study of protein folding and stability." *Biochemistry* 23, no. 8

electrophoresis method used by Cowman et al⁶³ to visualize oligomers of chondroitin sulfate was the method of choice for the present work.

Viscosity of Sodium Hyaluronate: Early Studies

Ogston and Stanier¹⁰ observed that the shear elasticity of synovial fluid might be significant with respect to joint function. Hyaluronate when compared to other polysaccharides under similar solvent conditions and identical molecular weight (1×10^6), shows a higher intrinsic

(1984): 1888-1894.

⁶³ Cowman, M. K., M. F. Slahetka, D. M. Hittner, J. Kim, M. Forino, and G. Gadelrab. "Polyacrylamide-gel electrophoresis and Alcian Blue staining of sulphated glycosaminoglycan oligosaccharides." *Biochemical Journal* 221, no. 3 (1984): 707-716.

viscosity and greater expanded coil dimensions. When compared with the Kratky-Porod (KP) worm-like model and the helical worm like chain model (HW), hyaluronate is seen as a hydrodynamic worm-like cylinder of about 1.1 nm. Some stiffness between nearest neighbors through hydrogen bonding is suggested.⁶⁴ In fact, Scott and Tigwell^{64 33} suggested the possibility of inter-residue hydrogen bonding to account for the observed stiffness of hyaluronate.

⁶⁴ Scott, J. E., and M. J. Tigwell. "The influence of the intrapolymer environment on periodate oxidation of uronic acids in polyuronides and glycosaminoglycuronans." (1975): 662-664.

Barrett and Harrington⁶⁵ studied flow birefringence and viscosity changes in hyaluronate solutions as a function of pH. They used concentrations of HA ranging from 0.25 to 0.0625% w/v at pHs 6.6, 7.5, 8.0, and 8.5 at constant ionic strength (0.1). The authors found dramatic changes in the viscosity and optical anisotropy as a function of pH. They reasoned that the abrupt changes observed in the physiological range might be indicative of a structure transition of the cooperative type.

⁶⁵ Barrett, T. W., and R. E. Harrington. "Low velocity gradient flow birefringence and viscosity changes in hyaluronate solutions as a function of pH." *Biopolymers* 16, no. 10 (1977): 2167-2188.

Viscosity studies of HA show a molecular weight dependence on viscosity. For $M > 10^5$, a fit for equation 1 would depend on salt concentration.³⁷

$$[\eta] = K M^a$$

Eq. 1

However, below $M = 10^5$, $[\eta]$ falls below the expected value probably because of non-Gaussian behavior of short chains. Shimada and Matsumura²⁸ investigated the intrinsic viscosity of high and low molecular weight hyaluronate. They found that two double log plots of $[\eta]$ versus M_w intersects at 1.5×10^5 .

The breakdown of this molecular weight is in agreement with the results of Cleland.³⁷ The "a" value derives from the slopes of these plots for

the most part, is related to the shape and flexibility of the polymer.^{28 37} Judging from the viscosity data, ²⁸ there is an increase in chain flexibility between 20,000 and 60,000 Daltons (g/mole and a leveling off between 10^5 and 2×10^5 corresponding to a Gaussian coil.

Balazs⁶⁶ studied the sedimentation volume of hyaluronate under various conditions of pH, ionic strength, and cation type.

The author found a decrease in the sedimentation volume of the hyaluronate with increasing salt concentration. Mg was found to

⁶⁶ Balazs, ENDRE A. "Sediment volume and viscoelastic behavior of hyaluronic acid solutions." In *Federation proceedings*, vol. 25, no. 6, p. 1817. 1966.

be more effective than sodium in decreasing the sedimentation volume. In aqueous solution of hyaluronate at pH 2.5, a non-linear relationship exist between the HA concentration in solution and that found in the sediment. It was further observed that the unusual and the unexpected rheological properties are exhibited by HA at this pH and at $I/2$ greater than 0.01. The entire sediment can be moved as a single piece, broken and put back together again like putty. This “putty-like” viscoelastic gel does not appear to be cross-linked, but form intermolecular associations controlled by the amount of charge on the polymer suggestive of an electrostatic force.

The rheological properties of HA putty have

been extensively studied.^{22 67 68} The dynamic viscoelastic properties of HA solutions have been measured over the frequency range of 0.02 to 1.67 cps by Gibbs and others.⁶⁹ The authors studied the effects of temperature, concentration, pH, and ionic strength on the dynamic shear moduli.

⁶⁷ Welsh, E. J., D. A. Rees, E. R. Morris, and J. K. Madden. "Competitive inhibition evidence for specific intermolecular interactions in hyaluronate solutions." *Journal of molecular biology* 138, no. 2 (1980): 375-382.

⁶⁸ Morris, Edwin R., David A. Rees, Geoffrey Robinson, and Graham A. Young. "Competitive inhibition of interchain interactions in polysaccharide systems." *Journal of molecular biology* 138, no. 2 (1980): 363-374.

⁶⁹ Gibbs, David A., E. W. Merrill, K. Af Smith, and E. A. Balazs. "Rheology of hyaluronic acid." *Biopolymers* 6, no. 6 (1968): 777-791.

The data showed that G' and G'' curves diverge very rapidly and extend over a very narrow frequency range as compared to other polymers such as polystyrene. This observation and the absence of a plateau region in the G' region and a minimum in the G'' curve tends to lend credibility to the idea that entanglement coupling in HA does not play a significant role in its observed rheological properties. Chain-chain association without entanglement could mean that a transient network structure exists in HA, which could be easily disrupted by the “melting out of these intermolecular associations upon

heating”.⁷⁰

Theoretical Considerations

Hyaluronate in solution behaves like a random coil polyelectrolyte with some stiffness. This stiffness might be the result of inter-residue hydrogen bonding or chain-chain association, which can be enhanced in the presence added salt or lowering the pH. Therefore, the charged polymer has charged counter-ions and byions in solution, which must be considered when making viscosity measurements. The expansion

⁷⁰ Morris, Edwin R., David A. Rees, and E. Jane Welsh. "Conformation and dynamic interactions in hyaluronate solutions." *Journal of molecular biology* 138, no. 2 (1980): 383-400.

of polyelectrolyte coils cannot be interpreted in the absence of salt because of the electroviscous (the lag of the counter-ion behind the charged polymer moving through a fluid) as well as the inability to eliminate particle interaction at zero concentration.

For uncharged particles, we can extrapolate $[\eta]_{sp}/c$ to zero concentration and eliminate the effects of particle interaction. One approach to solving this problem is to dialyze the desired sample in the appropriate salt solution and use the dialysate as the diluent. In this way, you can maintain charge balance at every dilution. This

method known as isoionic dilution⁷¹ was the method adopted by this author.

In a theta solvent, in which the shape of the polymer chains is described by the random flight model, the intrinsic viscosity, $[\eta]$, should be proportional to $M_2^{1/2}$. However, the dependence of the molecular expansion coefficient, α , on molecular weight can be approximated by $\alpha^3 \sim M^{a'}$ where $a = 1/2 + a'$ and α increases with molecular weight. Also the better the solvent, the larger the exponent a' and the larger a .⁷²

⁷¹ Pals, D. T. F., and J. J. Hermans. "Rec. trav. chim.", 71, 433 (1952).

⁷² Flory, Paul J. *Principles of polymer chemistry*. Cornell University Press, 1953.

In reality, polymers are stiffer than predicted for random coils⁷³ and if we disregard the effects of intramolecular interactions on chain configuration, the intrinsic viscosity would be proportional to the first power of the molecular weight and the coils would be free draining.⁷⁴

In solvent media better than theta solvents, the intrinsic viscosity of a linear polymer may be approximated by the empirical relationship: $[\eta] = K \times M^a$ and the exponent should be larger than

⁷³ Debye, Peter, and Arthur M. Bueche. "Intrinsic viscosity, diffusion, and sedimentation rate of polymers in solution." *The Journal of Chemical Physics* 16, no. 6 (1948): 573-579.

⁷⁴ Morawetz, Herbert. *Macromolecules in solution*. Vol. 21. New York: Interscience Publishers, 1965: 299-314.

0.5. In fact, the intrinsic viscosity is predicted to be proportional to $M^{0.8}$ in good solvent.

In the present work, the value of "a" is 0.8, k is 0.029 and $[\eta]$ is in units of ml/g. ^{37 75}

With respect to the intrinsic viscosities obtained for solutions of very short chain polymers, there is much more uncertainty. However, the value of the exponent, a, is expected to increase as plots of $\log [\eta]$ versus $\log M_2$ should give increasing slopes in the low molecular weight range. This is due to increased permeability of molecular coils.⁷⁴

⁷⁵ BALAZS, Endre Alexander. *The Amino Sugars. The Chemistry and Biology of Compounds Containing Amino Sugars...* Edited by Endre A. Balazs... and Roger W. Jeanloz. New York & London, 1965

The viscosity of solutions of flexible coil polymers at finite concentrations is represented by

$$\eta_{sp} = [\eta] + k' [\eta]^2 C$$

Eq. 2

where the Huggins constant k' , is independent of the molecular weight, but increases from values around 0.35 in strong solvent media to as high as 1.4 for solutions in very poor solvents.

The reduced viscosity, η_{sp} , is plotted versus concentration and the slope of the curve obtained is $k' [\eta]^2$. k values obtained from the slopes of small oligomers are determined and compared with the k obtained from the polymer.

Average molecular weights can be obtained from viscosity measurements using the relationship:

$$[\eta] = K \times \bar{M}_v^a$$

Eq. 3

Consider the expression⁷² :

$$\bar{M}_v = \left[\sum w_i M_i^a \right]^{1/a}$$

Eq. 4

where $w_i = C_i/C$ is the weight fraction of species i in the whole polymer. Hence,

$$[\eta] = K \times \bar{M}_v^a$$

Eq. 5

can be used in place of the formal equation for molecularly heterogeneous polymers

The simplest experimental method for the determination of viscosity is the measurement of the time; t , required for the passage of a volume, v , of solution through a capillary of length, l , with a cross-sectional radius, r . The relationship between $[\eta]$ and t are given by

$$\eta_{sp} = At + Bt$$

Eq. 6

where ρ is the density of the liquid and A and B depend on the dimensions of the capillary.⁷⁶

⁷⁶ Pearce, EM, C. Wright, and B. Bordoloi. "Competency based experiments in polymers." In *Abstracts of Papers of the American Chemical Society*, no. April, pp. 81-81. 1155 16th St, NW, Washington, DC 20036: American Chemical Society, 1979.

Circular Dichroism of HA Oligomers: Dependence on Chain Length-Theoretical Considerations

The conformation of a macromolecule can affect its optical activity.⁷⁷ Interaction between neighboring chromophores can lead to perturbations in the observed absorption spectra. Optically active samples can alter the properties of transmitted light by circular dichroism and circular birefringence.

Plane polarized light can be pictured as having two components: a left-handed and a right-handed component. The projection of each

⁷⁷ Cantor, C. R., and P. R. Schimmel, "Ultracentrifugation." *Biophysical Chemistry, Part II*. Freeman, New York (1980): Chapter 8, 409-433.

component of the light when viewed in this way would resemble a circle when projected onto a plane perpendicular to the direction of propagation. However, the refractive indices of a molecule from left and right-handed circular polarized light can differ. When this happens, one component is slowed with respect to the other component. In the wavelength region of the absorption band one component is also preferentially absorbed. The result is elliptically polarized light rather than plane polarized. Ellipticity is defined as the arc tangent of the ratio of the minor axis of the ellipse to the major axis. This is the quantity of interest.

Circular dichroism measures the differential absorbance of light by the sample over the

desired wavelength.⁷⁷ The relationship between CD and ellipticity is given by:

$$\Theta_{\lambda} \text{ (degrees)} = 2.303 (A_{1\lambda} - A_{r\lambda}) 180/4\pi$$

Eq. 7

The relationship between molar ellipticity and ellipticity is:

$$[\theta] = 100 \theta / Cl$$

Eq. 8

where A_l is the absorbance of the left hand component of the circular polarized light and A_r is the absorbance of the right hand component.

From the relationship:

$$A_l = \epsilon C l \quad A_r = \epsilon C l$$

Eq. 9

ϵ is the extinction coefficient, C is the concentration in moles per liter, and l is the path length in cm. $[\theta]$ is the molar ellipticity which allows for the determination of ellipticity independent of sample concentration. The units are $\text{deg cm}^2 \text{ dmol}^{-1}$.

ORD, optical rotary dispersion, is related to CD through a set of integrals called the Kronig-Kramers transforms. The optical rotation, θ , is given by:

$$\Phi (\text{degrees}) = 180 (\eta_l - \eta_r) / \lambda$$

Eq. 10

where l is the path length, $\eta_l - \eta_r$ are the refractive indices of the left and right hand components of circular polarized light,

respectively. λ is the wavelength of rotation. $[\Phi]$, the molar rotation is given by:

$$[\Phi] = 100\Phi/C$$

Eq. 11

If the ORD is known at all wavelengths, one can calculate the CD and vice versa. The ORD curve is a dispersion curve while the CD resembles an absorption curve.

The magnitude of the CD absorption is called the rotational strength and can be either negative or positive.⁷⁷ According to the coupled oscillator theory, CD bands arise from three terms. For a dimer, the one electron term is small and the electromagnetic coupling term is small while the third term almost exclusively contributes to the absorption.

The third term is known as the coupled oscillator or the exciton term. The value of this term depends on the two chromophores and molecular geometry. The CD vanishes for many conformations of the dimer. It will be zero if both chromophores are co-planar, parallel, or perpendicular to each other. In the course of this work, our interest is in electronic transitions from the carboxylate and amide moieties found in hyaluronic acid dimer, oligomers, and polymer.

Early Studies

CD and ORD studies were first performed on

mucopolysaccharides and mucopolysaccharides – dye complexes by Stone.⁷⁸ In this work a cotton effect was observed in the $\pi \rightarrow \pi^*$ and the $n \rightarrow \pi^*$ amide transition region. All mucopolysaccharides showed a negative band between 208 and 211 nm due to the 3 \rightarrow 1 glycosamine sugar, while the 4 \rightarrow 1 linkage gave rise to bands centered around 190 nm. The glycosidic linkage affects the magnitude and position of the CD bands.^{78 79 80}

⁷⁸ Stone, Audrey Larack. "Optical rotatory dispersion of mucopolysaccharides and mucopolysaccharide-dye complexes." *Biopolymers* 3, no. 6 (1965): 617-624.

⁷⁹ Cowman, Mary K., C. Allen Bush, and Endre A. Balazs. "Vacuum-ultraviolet circular dichroism of sodium hyaluronate oligosaccharides and polymer

In a study of the importance of linkage to the CD of glycosaminoglycans and gangliosides in water, two bands in the region of the amide and the carboxyl transitions were observed. The longest wavelength band was common to all polymers containing the amino sugar derivative, whereas a second band was linkage dependent. 4 → 1 linked amino sugars gave an additional positive band at approximately 185 nm.

In mixed solvents (water/ethanol), the increased negative band seen (compared to aqueous solution) below 200 nm was decidedly

segments." *Biopolymers* 22, no. 5 (1983): 1319-1334.
⁸⁰ Rees, D. A., *J. Chem. Soc.* 8 (1970): 877-884

due to hydrogen bonding across the β (1 \rightarrow 4 glycosidic linkage involving the amide transition. The large rotational strength seen for the polymer under conditions, which favor interchain association, might reflect a preferred orientation, which facilitates hydrogen bonding. Hydrogen bonding to the carboxylate group is likely since there is an increase in absorption in this region with decreasing pH.^{81 82 83}

⁸¹ Chakrabarti, B., and E. A. Balazs. "Optical properties of hyaluronic acid: Ultraviolet circular dichroism and optical rotatory dispersion." *Journal of molecular biology* 78, no. 1 (1973): 135-141.

⁸² Park, Jf W., and B. Chakrabarti. "Optical properties and viscosity of hyaluronic acid in mixed solvents: Evidence of conformational transition." *Biopolymers* 17, no. 5 (1978): 1323-1333.

⁸³ Buffington, Lynn A., Eugene S. Pysh, Bireswar Chakrabarti, and Endre A. Balazs. "Far-ultraviolet

Cowman and others, on the other hand, working with oligomers in neutral aqueous solution reported nearly equal and opposite sign contributions not seen in the polymer due to a cancellation effect.

These data were interpreted using a using an “end effect” model where the interior residues were treated like that of the polymer segment residues and contributions to the high energy transitions seen below 200 nm were attributed to the end residues and were found to be linkage

circular dichroism of N-acetylglucosamine, glucuronic acid, and hyaluronic acid." *Journal of the American Chemical Society* 99, no. 6 (1977): 1730-1734.

dependent.^{54 79} An end residue sequence NaGlcUA (β 1 \rightarrow 3) GlcNAc, gives a negative band at 190 nm with a molar ellipticity of -24000 deg cm²/dmol⁻¹. A reversed sequence, (β 1 \rightarrow 4) NaGlcUA, gives a positive band centered at 192 nm. A molar ellipticity of +14000 was found. A cooperative stabilization of an ordered conformation was not discernible from this data.

Studies on oligomers have been limited to small oligosaccharides. A method has been developed where oligosaccharides of various chain lengths can be studied using CD. In this way, the effect of chain length and solvent on circular dichroism of HA oligomers under physiological and perturbing conditions can be assessed. The author is interested in these

effects since chain-chain association in HA is an important aspect of its function and is thought to be responsible for the changes seen in previous CD studies. A minimum chain length might be required for a conformational transition to occur leading to a CD spectrum indistinguishable from that of the polymer.

Light Scattering of HA Oligomers: Introduction

Hyaluronic acid is a linear poly-dispersed polymer with alternating β - D - glucuronate and N-acetyl β - D - glucosamine residues. The polymer is poly-disperse in nature having a molecular weight between 14×10^6 and 77×10^3 .

⁸⁴ ⁸⁵ Human umbilical cord HA was studied by Laurent and Gergely³⁸ using a Brice-Speiser light scattering instrument at a wavelength of 4358 angstrom. They measured the scattered light between 15 and 135 degrees with respect to beam direction. HA was found to behave like a random coil with a molecular weight somewhere between 2.8×10^6 and 3.0×10^5 . Its radius of gyration was estimated at 220 nm when calculated from a partially rigid coil model. Low angle x-ray and viscosity data⁶⁸ indicates a

⁸⁴ Park, J. W., and B. Chakrabarti. "Solvent induced changes in conformation of hyaluronic acid." *Biopolymers* 16, no. 12 (1977): 2807-2809.

⁸⁵ Laurent, Torvard C. "Structure of hyaluronic acid." *Chemistry and molecular biology of the intercellular matrix* 2 (1970): 703-732.

persistence length of about 4 nm in 0.2 M NaCl.

Silver and Swann carried out laser light scattering measurements on low molecular weight vitreous HA and high molecular weight rooster comb HA.⁸⁶ The authors noted a smaller second virial coefficient for the lower molecular weight species as compared to the high molecular weight polymer. Sheehan and Phelps⁸⁶ reported that the nature of the counter ion has a profound effect on the apparent molecular weight and the viscosity of HA in solution. They

⁸⁶ Sheehan, J. K., C. Arundel, and C. F. Phelps. "Effect of the cations sodium, potassium and calcium on the interaction of hyaluronate chains: a light scattering and viscometric study." *International Journal of Biological Macromolecules* 5, no. 4 (1983): 222-228.

reported that the molecular weight of NaHA was twice that of KHA.

However, Mansson et al,⁵⁹ using two different molecular weight preparations from rooster comb HA and using low angle laser light scattering, observed no such effect.⁵⁹ Wik et al ⁸⁷ used high and low angle light scattering, and gel chromatography to determine the molecular weight distribution of HA samples. The authors obtained a relationship between the intrinsic viscosity and the molecular weight, which confirms the random coil structure of the polymer in dilute solution.

⁸⁷ Wik, K.O. (1979) Doctoral Thesis, Faculty of Medicine, Uppsala, Sweden.

Cleland and Wang carried out molecular weight measurements by Osmometry, light scattering, and sedimentation diffusion. They determined the Mandelkern-Flory parameter, β , for samples of HA ranging in Mw from 3×10^4 to 1.1×10^6 . These results confirm the flexible coil nature of the polymer.

Experimental Objective

The experimental objective of this work rests on the discovery that certain cationic dyes could bind hyaluronic acid oligomers in a chain length dependent way, with oligomers containing less

than 7 disaccharides not binding dye at all.⁸⁸

This discovery raised the question of the importance of hyaluronic acid chain length with respect to the unusual rheological properties shown by the polymer both in biological tissue and in solution. Intramolecular and intermolecular association of the polymer segments might involve complex equilibria between different conformations of the polymer. These conformations may be in part, governed by the length of the interacting segments.

⁸⁸ Turner, Raymond E., and Mary K. Cowman. "Cationic dye binding by hyaluronate fragments: dependence on hyaluronate chain length." *Archives of biochemistry and biophysics* 237, no. 1 (1985): 253-260.

In this work, hyaluronic acid fragments of various degrees of polymerization are produced and their physical properties are examined both as isolated chains and in combination with the polymer. It was hoped that a study of the chain length dependency of physical properties could lead to a proposed model for hyaluronic acid behavior in solution and in vivo.

Chapter 2: Cationic Dye Binding by Hyaluronate Fragments: Dependence on Hyaluronate Chain length

Introduction

Hyaluronate is a high molecular weight glycosaminoglycan from connective tissue, which is composed of alternating N-acetyl- β -D - glucosamine and β - D - glucuronic acid residues linked at the 1,3 and 1,4 positions, respectively.⁸⁹ The overall conformation of the polymer in aqueous solution resembles that of a

⁸⁹ Meyer, Karl. "Chemical structure of hyaluronic acid." In *Federation proceedings*, vol. 17, no. 4, pp. 1075-1077. 1958.

random coil with some stiffness.^{83 90} The stiffness results from the limited conformational freedom about the glycosidic linkages.^{91 92 93} Although evidence of inter-residue hydrogen bonding has been presented in the literature,³³

⁹⁰ Balazs, E. A. "Physical chemistry of hyaluronic acid." In *Federation proceedings*, vol. 17, no. 4, pp. 1086-1093. 1958.

⁹¹ Rees, D. A. "Conformational analysis of polysaccharides. Part II. Alternating copolymers of the agar-carrageenan-chondroitin type by model building in the computer with calculation of helical parameters." *Journal of the Chemical Society B: Physical Organic* (1969): 217-226.

⁹² Cleland, Robert L. "Ionic polysaccharides. V. Conformational studies of hyaluronic acid, cellulose, and laminaran." *Biopolymers* 10, no. 10 (1971): 1925-1948.

⁹³ Potenzzone, R., and A. Hopfinger. "Conformational analysis of glycosaminoglycans. III. Conformational properties of hyaluronic acid and sodium hyaluronate." *Polymer Journal* 10, no. 2 (1978): 181-199.

³⁴ ⁹⁴ ⁹⁵ ⁹⁶ ⁹⁷ the amide proton may hydrogen bond preferably to solvent water.³⁵

Intermolecular or intramolecular association of

⁹⁴ Scott, J. E., and F. Heatley. "1H nuclear-magnetic-resonance spectra of the methyl group of the acetamido moiety and the structure of acid glycosaminoglycans in solution." *Biochemical Journal* 181, no. 2 (1979): 445-449.

⁹⁵ WELTI, David, David A. REES, and E. Jane WELSH. "Solution Conformation of Glycosaminoglycans: Assignment of the 300-MHz 1H-Magnetic Resonance Spectra of Chondroitin 4-Sulphate, Chondroitin 6-Sulphate and Hyaluronate, and Investigation of an Alkali-Induced Conformation Change." *European Journal of Biochemistry* 94, no. 2 (1979): 505-514.

⁹⁶ Scott, John E., and Frank Heatley. "Detection of secondary structure in glycosaminoglycans via the 1H nmr signal of the acetamido NH group." *Biochemical Journal* 207, no. 1 (1982): 139-144.

⁹⁷ Heatley, Frank, John E. Scott, Roger W. Jeanloz, and Evelyne Walker-Nasir. "Secondary structure in glycosaminoglycuronans: NMR spectra in dimethyl sulphoxide of disaccharides related to hyaluronic acid and chondroitin sulphate." *Carbohydrate research* 99, no. 1 (1982): 1-11.

chain segments may contribute to their stiffness. Rheological studies have been performed on hyaluronic acid polymers and some interesting results have been obtained.

For example, Balazs (1966), Gibbs et al (1968), Welch et al (1980) and Morris (1980) performed such studies and found that hyaluronic acid could form putty at low pH in water and a rigid gel at low pH in ethanol-water mixtures. ^{66 67 69 70} In human eye vitreous, hyaluronic acid is involved in frictional interaction with collagen fibers and is essential to the stabilization of the micro-architecture of

the human eye vitreous.⁹⁸

One approach to the analysis of HA conformation and self-association is to study the molecular weight dependence of physical properties. This approach is based on the idea that a minimum number of repeat units might be required for cooperative stabilization of ordered structures. It has been noted that HA fragments, studied in water at neutral pH, show molecular weight dependent circular dichroism and nuclear magnetic resonance properties.^{34 81}

⁹⁸ EA Balazs. The molecular biology of the vitreous. A McPherson (Ed.), New and Controversial Aspects of Retinal Detachment, Harper & Row, New York (1968), pp. 3–15

⁹⁴⁻⁹⁹ This dependence, however, is attributed to the distinct contributions of the end residues of short chains.³⁵⁻⁵⁴⁻⁷⁹

However, when the polymer is studied in solvent, which promotes gel formation, the polymer conformation is altered and this gel effect is not duplicated by a chemically degraded sample.¹⁰⁰ A molecular weight dependence has also been observed in the capability of HA

⁹⁹ Chakrabarti, B., and D. A. Brant. "Solution Properties of Polysaccharides." In *ACS Symposium Series*, vol. 150, p. 275. 1981.

¹⁰⁰ Park, Joon Woo, and Bireswar Chakrabarti. "Conformational transition of hyaluronic acid carboxylic group participation and thermal effect." *Biochimica et Biophysica Acta (BBA)-General Subjects* 541, no. 2 (1978): 263-269.

fragments to intermolecularly associate with HA polymer. The fragments have been shown to disrupt the network structure of the polymer gel and the chain length requirement appears to be between 4 and 60 disaccharides.⁶⁷

One additional property of HA which appears to be molecular weight dependent is the capability to bind and aggregate cationic dyes in neutral aqueous solution. High molecular weight HA binds acridine orange, resulting in exciton splitting, hypochromism, and induced optical activity in the visible region absorption band of the dye.^{20 101} A low molecular weight HA

¹⁰¹ Stone, Audrey L., and Dan F. Bradley.

preparation (approximately 12-13 disaccharides in length) does not aggregate acridine orange.¹⁰²

In the present study, we further examine the interaction between HA oligosaccharides and cationic dyes. By using polyacrylamide gels electrophoresis as the basis for a rapid screening method. As a function of chain length, the oligosaccharides show a dramatic change in dye binding.

"Aggregation of cationic dyes on acid polysaccharides I. Spectrophotometric titration with acridine orange and other metachromatic dyes." *Biochimica et Biophysica Acta (BBA)-General Subjects* 148, no. 1 (1967): 172-192.

¹⁰² Balazs, Endre A., ed. *Chemistry and molecular biology of the intercellular matrix*. Vol. 3. Academic Press, 1970.

Materials and Methods

Sodium hyaluronate was the purified preparation from rooster comb, previously described.⁵⁴ The protein content was less than 1% of the HA content. The molecular weight estimated from the limiting viscosity number in 0.15 M NaCl (2700 ml/gm), was 1.6×10^6 . Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35), with a specific activity of 14,000 USP/NF units/mg, was obtained from Worthington Biochemical Corporation.

Leech head hyaluronidase (hyaluronate 3-glycanohydrolase, EC 3.2.1.36) was the grade 1 material obtained from Fisher Scientific Company. Bio-gel P-30 (~400 mesh) for gel

filtration chromatography, Bio-Rad AG50WX resin for ion exchange, and all reagents for polymerization of electrophoretic gels were obtained from Bio-Rad Laboratories. Alcian Blue 8GS, acridine orange, azure A, and toluidine blue were obtained from Fluka Chemical Corporation. Stains-All was obtained from Bio-Rad Laboratories.

All other chemicals were of reagent grade quality.

Enzyme Digestion of Hyaluronate

The methods used in the digestion of HA by leech or testicular hyaluronidase were essentially as previously described.^{35 54 78} Specific details for the digestion employed in

direct comparison of chromatographic and electrophoretic profiles are given below.

A 200 mg quantity of HA was dissolved at 4 degrees centigrade in 20 ml 0.15M NaCl, 0.10M CH₃COONa, 0.001M Na₂EDTA, pH 5.0 (buffer A). Gelatin was dissolved in hot buffer A at a concentration of 1% (w/v), and stored overnight at 4 °C to gel. Bovine testicular hyaluronidase was dissolved immediate before use in cold buffer A at a concentration of 4700 units/ml.

The HA and gelatin solutions were pre-incubated at 37 °C for 1 hour prior to digestion. At t=0, 2.0 ml gelatin and 1.0 ml hyaluronidase was added to the hyaluronate. Incubation continued at 37 °C with slow stirring. At t=1 hour, an additional 1.0 ml aliquot of

hyaluronidase was added. At $t = 2.5$ hours, the digestion was terminated by heating in a boiling water bath for 10 minutes. The cooled digest was divided into three portions and frozen.

Gel-filtration and Oligosaccharide Isolation

One digest aliquot was rapidly thawed, while two 0.5 ml portions from the aliquot were refrozen for electrophoretic analysis. The thawed sample, containing approximately 60 mg HA fragments, was taken to dryness by rotary evaporation and redissolved overnight at 4 °C in 5 ml 0.5M pyridinium acetate, pH 6.5.

The sample was chromatographed on a 2.5 x 195 cm column of Biogel p-30 equilibrated in the same buffer at a flow rate of 3.4 ml/hr.

Fractions of 2.5 ml were collected and analyzed for uronic acid concentration by the automated carbazole method.¹⁰³ Appropriate fractions were pooled to yield purified oligosaccharide samples containing between 2 to 15 disaccharide repeat units. The oligosaccharides were dried, freed of pyridine, and converted to the sodium salt as previously described.⁵⁴ samples were stored frozen in aqueous solution at a concentration of 1.0 mg/ml.

The chain length of the smallest

¹⁰³ Balazs, E.A., Berntsen, K.O., Karossa, J. and Swann, D.A., 1965. An automated method for the determination of hexuronic acids. *Analytical biochemistry*, 12(3), pp.547-558.

oligosaccharides were determined from the molar ratios of uronic acid residues to reducing terminal N-acetylhexosamine, quantified by the Reissig et al¹⁰⁴ modification of the Morgan and Elson¹⁰⁵ method.

The tetrasaccharide and hexasaccharide gave molar ratios of 2.0 (theory, 2.0) and 3.1 (theory, 3.0), respectively. Larger oligosaccharides were identified by counting peaks in the

¹⁰⁴ Reissig, José L., Jack L. Strominger, and Luis F. Leloir. "A modified colorimetric method for the estimation of N-acetylamino sugars." *Journal of Biological Chemistry* 217, no. 2 (1955): 959-966.

¹⁰⁵ Morgan, Walter Thomas James, and Leslie Alderman Elson. "A colorimetric method for the determination of N-acetylglucosamine and N-acetylchondrosamine." *Biochemical Journal* 28, no. 3 (1934): 988.

chromatographic profile previously shown to have a one to one correspondence with species of increasing chain length.⁵⁴

Polyacrylamide Electrophoresis

The procedure for analysis of digest mixtures and oligosaccharides by vertical slab gel electrophoresis has been described elsewhere.⁶³ The 15 x 14 x 0.25 cm polyacrylamide gels contained 10% polyacrylamide, 0,33% N,N'-methylenebisacryamide in 0.1M Tris-borate, 0.001 Na₂EDTA, pH 8.3. Digest samples in the original digestion buffer, or purified oligosaccharides in water were mixed with 1/10 volume 2M sucrose in Tris-borate-EDTA buffer and applied directly to the gel. The difference in

salt and buffer concentrations between these samples has previously been shown¹⁰⁶ to have a negligible effect on mobility in this procedure.

Digest samples containing up to 300 μg or purified oligosaccharides containing up to 40 μg were electrophoresed at 20 mA (125 V) for 20 minutes and 40 mA (250 V) for 2 hours until the bromophenol blue tracking dye reached approximately 3.0 cm from the bottom of the gel.

The usual staining procedure utilized a 0.5 % solution of alcian blue in water. Gels were

¹⁰⁶ Knudson, Warren, Mary W. Gundlach, Thomas M. Schmid, and H. Edward Conrad. "Selective hydrolysis of chondroitin sulfates by hyaluronidase." *Biochemistry* 23, no. 2 (1984): 368-375.

immersed in this solution for 45 minutes, and destained in water. In order to quantitate the stained pattern, gels were scanned at 615 nm on a Kontes Model 800 scanning densitometer. For direct comparison of dye binding reproducibility, linearity with load, and chain length dependence, purified oligosaccharide species were electrophoresed in adjacent lanes of a single gel.

Peaks from the densitometric scans of purified oligomers were cut out and weighed as a measure of relative peak areas. Staining with other dyes or solvent systems employed the same general procedure, but the destaining solvent was identical to the solvent used to dissolve the particular dye. Gels stained with

acridine orange were scanned at 450nm.

Limited Enzymatic Digestion of Sodium Hyaluronate: Comparison of Gel Filtration and Electrophoretic Patterns

Incomplete digestion of high molecular weight HA with bovine testicular hyaluronidase yields a mixture of oligosaccharides. The oligosaccharides vary in chain length, but have a common structural pattern^{29 104} of β -D-GlUA p-[1 \rightarrow 3)- β -D-GlcNAc p-(1 \rightarrow 4)- β -D-GlcUA p]_{n-1} - (1 \rightarrow 3)- β -D-GlcNAc (see Figure 1).

As previously reported, the oligosaccharides containing up to approximately 15 disaccharide units may be separated by gel filtration chromatography on Bio-Gel P-30. The

chromatographic profile of such a digest is shown in Figure 3.

Although gel filtration is an excellent technique for the isolation of purified oligosaccharides, polyacrylamide electrophoresis is more suitable for analytical purposes.^{63 63 107} In this method, oligosaccharides with a common charge to mass ratio are separated based on molecular size by the sieving effect of the polyacrylamide matrix. The smaller the oligosaccharides are, the greater the mobility.

¹⁰⁷ Hampson, I. N., and John T. Gallagher. "Separation of radiolabelled glycosaminoglycan oligosaccharides by polyacrylamide-gel electrophoresis." *Biochemical Journal* 221, no. 3 (1984): 697-705.

Figure 4 shows the results of polyacrylamide electrophoresis of a 2.5 hr. digest of HA.

The corresponding chromatographic profile is shown in Figure 3. When the sample is stained with alcian blue in water, 25-30 discrete bands can be discerned. The individual bands were identified by running purified oligosaccharides obtained from column chromatography in lanes adjacent to the digest samples. Each band in the digest mixture corresponds to a single oligosaccharide species, with adjacent bands differing by one disaccharide unit in length.

The smallest HA oligosaccharide which could be detected in the electrophoretic pattern of the digest mixture contains seven disaccharide units. A densitometric scan of the stained gel is

given in Figure 5. There are clear differences between this profile and the chromatographic patterns of Figure 3. Oligosaccharides smaller than $n=7$ are not detected in the stained gel. Species with $n=7$ to $n=11$ show an increasing sensitivity to stain and species with $n > 11$ appear to stain in approximately correct proportion to their concentrations.

Essentially identical results are obtained for total sample loads of 200 of 500 micrograms. Variations in duration of staining (30 minutes To 3 hours) or destaining (5 min. To several days) did not alter the results. Enzymatic digestion of HA with leech hyaluronidase yields the same pattern of chain length dependent staining. (It should be noted that impure preparations of HA

when digested with testicular hyaluronidase show additional faster moving bands. These species are oligosaccharides of sulfated glycosaminoglycans and/or their transglycosylation products with HA as seen in Figure 4.

The chain length dependence of HA oligosaccharide staining is unlikely to be due solely to differences in the rate of diffusion from the gel matrix. Chondroitin 4 - sulfate, Chondroitin 6- sulfate, and dermatan sulfate oligosaccharides containing only three repeat units are easily visualized when stained with alcian blue in 2% acetic acid¹⁰³. Under these conditions, the HA pattern is similar to that obtained in water.

Quantitation of Alcian Blue Staining of Purified Oligosaccharides

HA oligosaccharides containing less than 7 repeat units may not be stained by alcian blue at sample loads up to 100 micrograms. However, larger oligosaccharides containing 8 to 15 (Figure 7) repeating units show a linear increase in alcian blue binding as a function of sample load, over the range 2-4 μg . The relative affinities of the different oligosaccharides for alcian blue were determined by loading identical quantities (30 or 15 μg) of each species on a single gel.

In agreement with the results for the unfractionated digest samples, a decrease in dye binding capability with decreasing chain length was found (

Figure 6). The $n=9$ species binds approximately 30 to 40% as much alcian blue as

the n=15 fragment. The n=8 species binds only about 20% as much alcian blue as n=15.

Dye Structure and Solvent Effects

The staining of HA oligosaccharides was examined with several different dyes to determine if the observed chain length dependence is unique to alcian blue interactions. The densitometric profile of a second unfractionated HA digest, stained with 0.5% alcian blue, 0.1% acridine orange, or 0.1% azure A (all in water) is shown in (Figure 7).

The profiles differ somewhat in the range of chain lengths over which dye binding capability varies, but all show negligible dye binding by oligosaccharides smaller than 7 repeat units.

Essentially identical results were obtained with 0.01% toluidine blue in water. The dye concentrations chosen for these studies were those affording the best contrast of stained dye relative to background. Variation of dye concentration from 0.1 to 0.5% (alcian blue), 0.05 to 1.0 % (acridine orange or azure A), or 0.01 to 0.1% (toluidine blue) showed no significant changes in the observed patterns.

The singly charged planar heterocyclic dyes such as acridine orange, azure A, and toluidine blue do not bind strongly to HA oligosaccharides in an acidic staining solvent containing 2% CH₃COOH. However, in comparison, the multiple charged copper phthalocyanine derivative, alcian blue, is capable of interacting

with residual charges on the HA in this medium (the pH within the gel during the staining is not known). The densitometric profile obtained under these conditions resembles that obtained in water, but shows about four fewer bands (Figure 7). Staining solvent composed of 20 % ethanol, 2% CH₃COOH leads to weak staining of HA oligosaccharides by alcian blue. Figure 8 shows the structure of the dyes used.

Stains-All, a cationic carbocyanine dye previously used to stain polymeric glycosaminoglycan, was employed as a 0.005% solution in water or in 5% formaldehyde at pH 7.3. The stained gel showed a diffuse pattern with no clear bands. Qualitative examination of the broad stained for purified n=8 and n=13

oligosaccharides in the formaldehyde - water staining solution showed a greatly reduced incorporation of stain by the smaller species relative to the larger species.

Discussion

Oligosaccharides can be separated on polyacrylamide gels to produce discrete bands where each band corresponds to a unique molecular weight species. In the present case, adjacent oligosaccharide bands differ in chain length by only one disaccharide unit. Precipitation of the oligosaccharide species with cationic dyes lead to the visualization of chain length dependence of dye binding. The ability of the oligosaccharides to bind dye increases with

increasing chain length, varying from about 12 to 30 disaccharides for maximal binding depending on dye type and solvent condition.

The interpretation of the chain length dependence involves the consideration of several factors. The nature of dye binding in the polyacrylamide matrix is not fully understood and the nature of dye interaction with HA must be considered. Other types of chain length dependent behavior should be investigated as well.

Oligosaccharide Staining within a Polyacrylamide Matrix

The staining of an Oligosaccharide within a polyacrylamide matrix involves two processes, dye binding and precipitation of the dye-

oligosaccharide complex. One potential artifact of the polyacrylamide assay is the possibility of rapid diffusion of small oligosaccharides from the gel prior to dye binding. Arguing against this effect is the known precipitability of low molecular weight ($n=2-3$) chondroitin sulfate oligosaccharides by alcian blue under the same conditions, It is almost certain that diffusion of oligosaccharides in the absence of dye occurs.

However, diffusion in the presence of dye represents a reduced tendency to bind dye. Dye binding by small HA oligosaccharides to an extent insufficient to cause precipitation may occur. Since no residual staining is observed for very high loads of a small oligosaccharide, it is likely that HA oligosaccharide species less than

7 disaccharides bind dye weakly or not at all under our assay conditions,

Dye - Hyaluronate Interactions

The binding of cationic dyes to glycosaminoglycans is stabilized by both electrostatic and dye stacking interactions.²⁰ ¹⁰²

¹⁰⁸ ¹⁰⁹HA is a relatively rigid polyanion with only one charged site per disaccharide repeating unit.

¹⁰⁸ Salter, Mary K., E. William Abrahamson, and W. Barton Rippon. "Spectroscopic properties of complexes of acridine orange with glycosaminoglycans. II. Aggregated complexes—evidence for long-range order." *Biopolymers* 15, no. 7 (1976): 1251-1265.

¹⁰⁹ Scott, J. E., G. Quintarelli, and M. C. Dellovo. "The chemical and histochemical properties of Alcian blue." *Histochemie* 4, no. 2 (1964): 73-85.

For the extended HA conformation, which predominates in solution, the linear charge density is lower than that required for site binding of monovalent counter-ions.^{12 30 110}

Cationic dyes, in contrast, are bound and aggregated by HA in aqueous solution.^{20 101 102}

Dye stacking interactions may therefore profoundly influence the stability of complexes of HA with cationic dye. That electrostatic interactions also contribute to stability of the complex has been established by the disruption

¹¹⁰ Cleland, Robert L. "Enthalpy of Mixing Glycosaminoglycans with Aqueous NaCl." *Biopolymers* 18, no. 11 (1979): 2673-2681.

of complexes at low pH or moderate Ionic strength.^{20 102}

The electrostatic interaction between HA and cationic dyes is reduced in the case of low molecular weight oligosaccharides, for which the total charge is smaller. One plausible explanation for the observation of marked chain length dependence in dye binding rests on this consideration alone. The transition in dye binding may represent the change from simple electrolyte to polyelectrolyte behavior.

Chakrabarti and Balazs²⁰ have suggested a requirement for chain - chain association in order to bind and stack molecules in an asymmetric array. Chain-chain dimerization results in an effective doubling of the linear

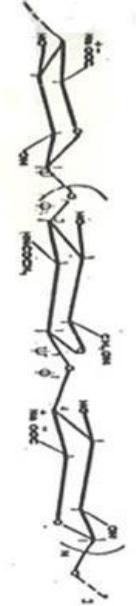
charge density of HA, and a potentially more favorable distribution of charged sites. This increases both dye stacking and electrostatic interactions. HA oligosaccharides, which bind dye, could therefore represent the smallest species capable of forming intermolecular associations.

Chain Length Dependent Behavior in HA

Welch et al⁶⁷ have demonstrated that low molecular weight oligosaccharides are incapable of forming intermolecular associations with polymeric HA in aqueous solution at neutral or low pH. The transition to species capable of chain association occurs within the size range of 4 to 60 disaccharides. The smallest HA fragment

showing CD spectral changes in salt-ethanol-acid solvent known to gel polymeric HA, is approximately 7 disaccharides in length (P. Staskus and W.C. Johnson, Jr., personal communication). The minimum chain length of HA which binds dye is seven disaccharides.

Repeat Unit of Sodium Hyaluronate



Sodium Hyaluronate

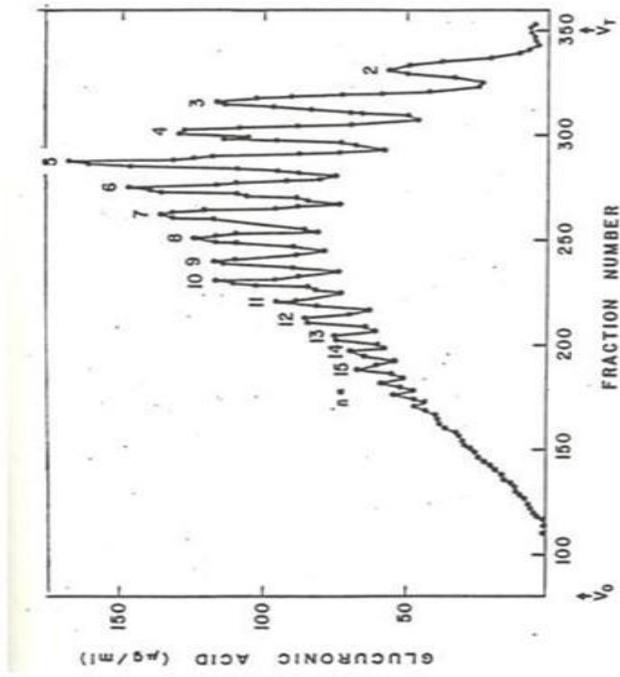
Figure 2. Repeat unit of sodium hyaluronate. Repeat is an N-acetyl β -D-glucosamine and a β -D-glucuronic acid linked at the 1, 3 and 1, 4 positions respectively. N represents the degree of polymerization.

HA, which binds dye, is seven disaccharides. Since the size ranges are similar and in consideration of the mechanism of HA-dye interactions, we propose that these phenomena are related. Chain association of HA fragments may exist within the polyacrylamide matrix prior to dye addition or may occur in part as a result of weak dye binding and resulting decrease in electrostatic repulsion between chains.

Whether the basis of the chain length dependent interaction of hyaluronate oligosaccharides with cationic dyes represents a simple a simple polyelectrolyte effect or a chain association phenomenon cannot be determined based on the data presented so far. However, oligosaccharides of higher weight average

molecular weights were produced and studied using viscometry and spectropolarimetry methods. These were performed to delineate the importance of molecular weight in the ability of polymeric HA to form network structures or cross links in-vivo and in-vitro.

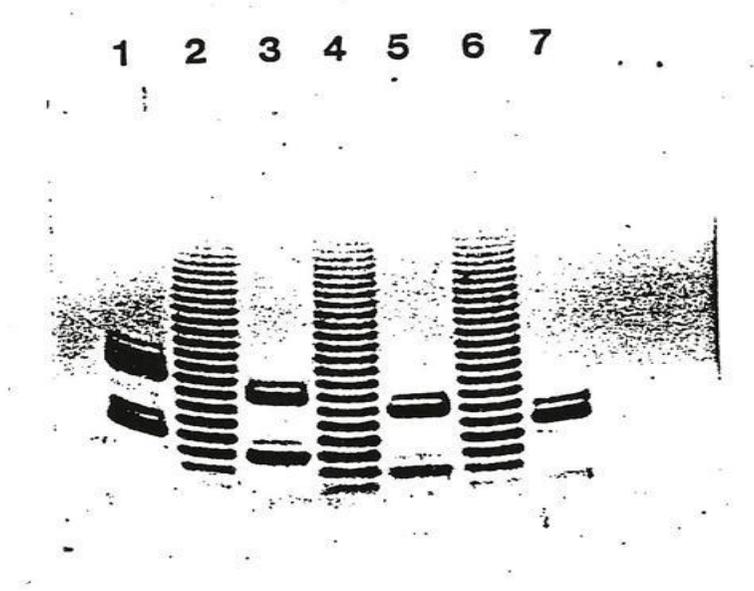
Gel filtration elution profile of HA oligomers produced by digestion with testicular hyaluronidase



Gel Filtration Profile

Figure 3. Gel filtration elution profile of HA oligomers produced by digestion with testicular hyaluronidase. Sample load was 60 mg. applied to a 2.5 x 195 cm Bio-Gel P-30 column (400 mesh) equilibrated with 0.5 M pyridinium acetate, pH 6.5. Numbers above peaks indicate the number of repeating disaccharide units.

Polyacrylamide Gel Electrophoresis of HA
Digested with Bovine Testicular
Hyaluronidase



Polyacrylamide Gel Electrophoresis

Figure 4. Polyacrylamide gel electrophoresis of HA digested with bovine testicular hyaluronidase (lanes 2, 4, 6) and purified HA oligosaccharides (lanes 1, 3, 5, 7). 200 μ g HA digest and 30 μ g of each purified oligosaccharide were electrophoresed. Lanes 1: n=15 and n=11, lane 3: n=14 and n=10, lane 5: n=13 and n=9, lane 7: n=12 and n=8. Gel was 10% polyacrylamide stained with 0.5% alcian blue in water.

Densitometric Scan of Gel Electrophoresis Pattern for HA digest Sample Containing a Mixture of Oligosaccharides

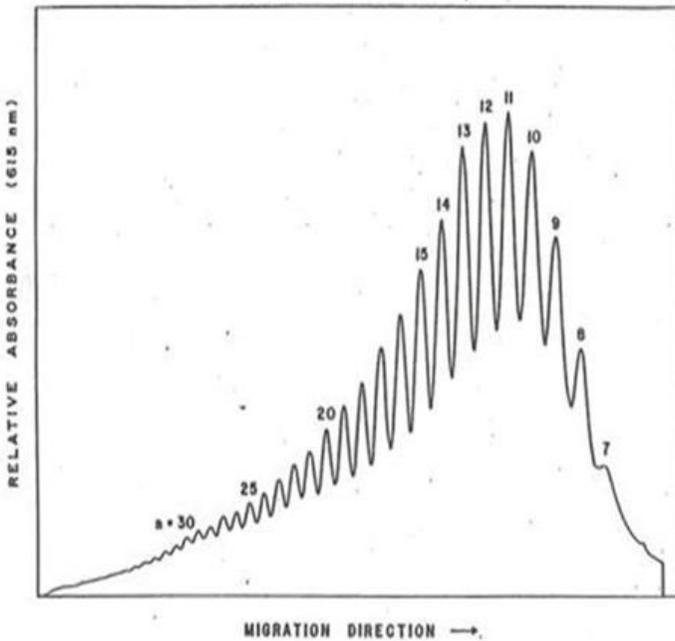


Figure 5. Densitometric scan of gel electrophoresis pattern for HA digest sample containing a mixture of oligosaccharides. Electrophoresis is in a 10% polyacrylamide gel stained with 0.5 % alcian blue in water. Sample load was 200 μg. The number of disaccharide repeats in each oligosaccharide is indicated.

Chain Length Dependence of Alcian Blue Staining for HA Oligosaccharides within a Polyacrylamide Gel

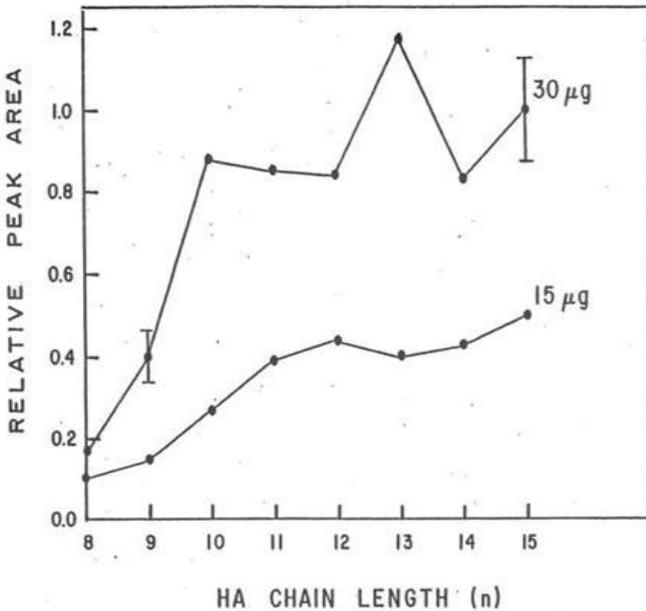


Figure 6. Plot shows chain length dependence of alcian blue staining for HA oligosaccharides within a polyacrylamide gel. Purified oligomers at sample loads of 15 and 30 µg were electrophoresed in adjacent lanes. Error bars indicate the range of observed values for a single oligosaccharide electrophoresed in 8 adjacent lanes of a single gel.

Comparison of Densitometric Profiles for an HA Oligosaccharide Mixture

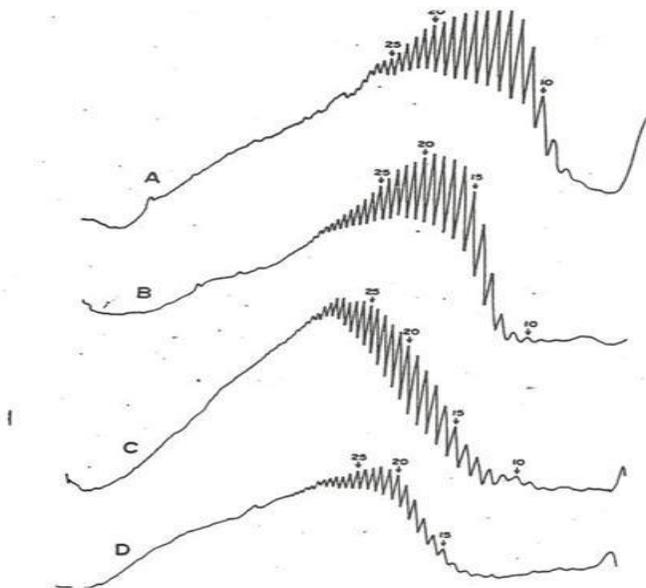


Figure 7. Comparison of densitometric profiles for an HA oligosaccharide mixture electrophoresed on 10 % polyacrylamide gel and stained with different dyes or solvent conditions; (A) 0.5% alcian blue in H₂O; (B) 0.1% acridine orange in H₂O; (C) 0.1% azure A in H₂O; (D) 0.5% alcian blue in 2% acetic acid. The number above the peaks indicates the number of repeat units.

Cationic dye structures used in the Staining of Polyacrylamide gels

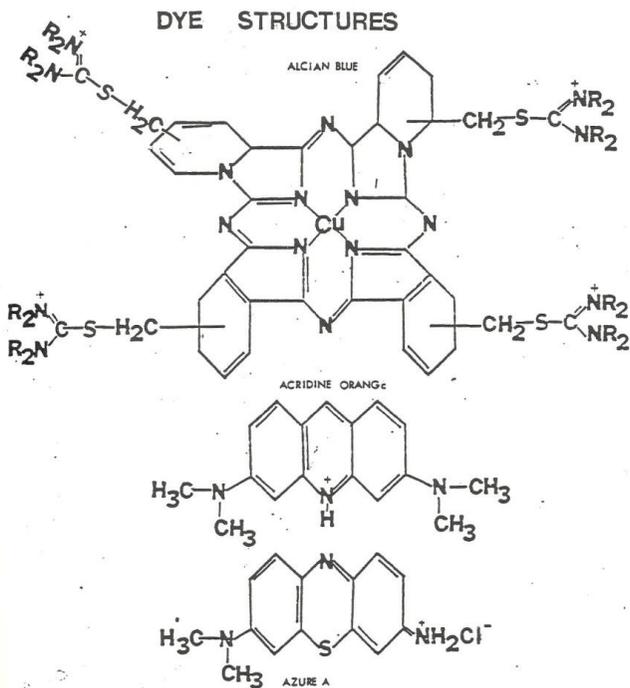


Figure 8. Cationic dye structures used in the staining of polyacrylamide gels.

Chapter 3: Intermolecular Interactions in HA Polymers: Effects of Added Oligomers

Introduction

Hyaluronic acid is a glycosaminoglycan composed of alternating β (1 \rightarrow 3)-D-glucuronate and N-Acetyl- β (1 \rightarrow 4)-D-glucosamine.⁸⁹ In solution, the polymer behaves as a stiff random coil polyions⁹⁰ ⁸⁶ and forms intermolecular associations, which are enhanced under the conditions of increasing Ionic strength, increasing concentration, decreasing pH and decreasing temperature.

It was previously reported that when equal concentrations of HA segments (~60

disaccharides) and high molecular weight HA are mixed, a once viscous solution of high polymer exhibits rheological properties resembling that of separated chains with no evidence of coupling.⁶⁷

The validity of these competitive inhibition experiments to characterize intermolecular junctions in polysaccharides has been studied⁶⁸ and rests on the idea that chains of sufficient length can successfully compete with longer chains in junction formation without cross linking and therefore would show a decrease viscosity. Silver and Swann³⁶ found a molecular weight dependence of the second virial coefficient from laser light scattering.

Balazs⁶⁶ investigated the rheological

properties of HA in solution and found that network formation in the polymer could be enhanced under certain conditions. NMR and hydrogen exchange studies of HA show the network structure of polymeric HA might be transient rather than static.^{32 88} Gibbs et al⁶⁹ studied the rheological properties of HA and found that no entanglement coupling of HA was evident over the concentration range of 2-4 mg/ml.

Increasing the Ionic strength or partially suppressing the charge at low pH reduces the coil dimensions and decreases the intrinsic viscosity of the polymer.⁷⁰ Under both neutral and acidic conditions, a temperature dependence of intrinsic viscosity has been

observed. There appears to be greater temperature dependence below 40 degrees. This indicates a lower activation energy requirement for network rearrangement at higher temperature. A "melting out of segmental interactions" was suggested as a probable cause.

In the present study, we prepared HA segments of various average chain lengths and added them to solutions of high polymer. We sought to determine if any of our segments are of sufficient length to uncouple high polymer through competitive inhibition in a manner analogous to that previously described by Rees and coworkers.⁶⁷ At $25 \pm 1^\circ \text{C}$ and at constant Ionic strength in 0.15 M NaCl solution, we wished to verify the existence of a chain length

dependent uncoupling of segmental interaction in polymeric HA and possibly identify a minimum chain length requirement for the formation of an ordered conformation in HA oligomers.

Materials and Methods

Sigma hyaluronate (grade 1, lot #1751) from human umbilical cord was obtained from Sigma Chemical Company and purified rooster comb HA (Healon) was a gift from Dr. Endre A. Balazs,

The Molecular weight estimated from the intrinsic viscosity is 1.8×10^6 in 0.15 M NaCl. Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, E.C.3.2.1.35), with specific activity of 4883 USP/NF units /mg was obtained

from Worthington Biochemical Corporation. DEAE-Sephadex anion exchanger (lot # 1B 30362) used in the purification of Sigma HA was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel P-60 was from BioRad corporation. All other chemicals were of reagent grade quality.

Purification of Sigma HA

Sigma HA was dissolved at 5 mg/ml in 0.3 M NaCl and allowed to hydrate for two days or until hydration was complete. The sample was passed through a DEAE-Sephacel anion exchange column (7cm x 1cm i.d.) previously washed with two volumes of 0.3 M NaCl. Yield was approximately 70%. The sample was eluted

under reduced pressure because of the high viscosity of the sample. The eluent (40 ml) was dialyzed against 6 liters of water at 4 degrees for 24 hours. The sample was then taken to dryness on a Brinkman Rotavapor (model R-110) and dissolved in 20 ml buffer A (1mM Na₂EDTA, 0.15 M NaCl, and 0.1 M CH₃COONa) and dialyzed against 4 liters of buffer A overnight.

*HA Segments: Isolation and Weight Average
Molecular Weight Determinations*

155 mg HA dissolved in 20 ml buffer A was allowed to incubate while slowly stirring at 37 °C. Freshly prepared testicular hyaluronidase (4395 units in 1 ml buffer A) was added to the HA and digestion proceeded for 30 minutes. The reaction was stopped by submerging the

reaction flask into boiling water for 10 minutes.

After cooling to room temperature, the sample was spun in a Sorvall preparative centrifuge at 10000 x g for 15 minutes. The salt concentration was adjusted. This was done by reducing the volume of the supernatant to approximately 1/3 to 0.5 M NaCl. A 0.5 ml sample was set aside for a polyacrylamide gel determination and the remainder of the sample applied to a Bio-Gel P-60 column (195 x 25 cm) at 15 ml/hr. Three ml fractions were collected. Glucuronate concentrations were determined for each fraction by the automated carbazole method.¹⁰⁴

A 10% polyacrylamide gel was run along with an oligosaccharide standard as previously described,⁸⁴ in order to determine which

fractions should be pooled. The pool fractions, which were of different molecular weight distributions, were dialyzed against 6 liters of water overnight, rotary evaporated to about 2-4 ml and then dialyzed against 0.15 M NaCl overnight. These samples were subsequently used in the viscosity experiments.

Further Characterization of HA Segments

The electrophoretic gels previously described, were scanned at 615 nm using a Kontes Fiber Optics Scanner (model number 800). The scanned profiles yielded peaks corresponding to each oligosaccharide (e.g. $N = 12, 13, 14$, etc.) in a particular pool and a standard of 12 or 13 disaccharides ($N = 12$ or 13). The distance from

the top of the gel to the center of each peak (peak migration distance) and the height of each peak relative to a common baseline affords a value of the weight average molecular weights since the molecular weight of each fraction is known from the standard. Four populations of segments were characterized in this manner. HA12 was prepared from two different digest preparations. HA12 (Figure 11) was prepared from a digest done two weeks after the first preparation (Figure 10) on HA of identical lot numbers.

Determination of Intrinsic Viscosity

Rooster comb HA ($[\eta] = 2900 \text{ ml/g}$) was prepared for viscosity measurements in 0.15 M

NaCl. The sample was allowed to hydrate in the solvent at 4 degrees centigrade for 2-3 days before dialyzing against 6 liters 0.15 M NaCl overnight. This dialysate was saved for use as the diluent. Sample concentration ranged from approximately 500-100 micrograms /ml HA.

HA segments were prepared for viscosity measurements by dialyzing the appropriate pooled samples (in 0.15 M NaCl) against 6 liters 0.15 M NaCl or 24 hours. The dialysate was saved for future use as diluent. Due to the low molecular weight of the segments, starting concentrations of up to 10 mg/ml were used.

When segments were added to the polymer, the mixture was allowed to stir slowly for 1/2 hour or 24 hours prior to taking measurements.

Nearly equal concentrations (700-500 -g/ml) and in most cases nearly equal volume ratio of polymer and segments were mixed.

Viscosity measurements were made in a Cannon-Ubbelohbe semi-micro dilution viscometer from Cannon Instrument Company. The temperature was maintained at 25 °C and sample concentration was determined at each dilution by taking aliquots directly from the viscometer. All samples were filtered through a 0.45-micron (Acrodisc) filter before measurements were taken.

Determination of Hyaluronic Acid Molecular Weight by Light Scattering

Eighteen ml of 0.15 M NaCl solution was poured over 2 ml Healon HA and the sample left

for 2-3days in the cold with gentle shaking until complete hydration was obtained. The sample was dialyzed against 0.15 M NaCl at 4 deg C overnight. Dilutions were made from the concentrated stock using the dialysate as diluent. Sample concentrations were determined on samples taken directly from the light scattering cell during the actual measurements. Samples were measured at room temperature with a Chromatix KMX-6 Low Angle Laser Light Scattering Spectrophotometer equipped with a He-Neon laser set at 632.6 nm. All measurements were done using the 6-7 degree annulus and the 0.2 mm field-stop. The samples were filtered through a 0.45-micron filter from Acrodisc (lot # 3299). The refractive index

increment used was that obtained by Wik et al (87) of 0.164 and the refractive index of the dialysate was taken to be 1.335.

Result: HA Segments Only

Hyaluronic acid oligosaccharides were prepared as described above and the distribution pattern visualized on a 10% polyacrylamide electrophoretic gel stained with alcian blue in water (**Error! Reference source not found.**). The gel was subjected to densitometric scans and the weight average molecular weight, number average molecular weight, and the range of chain sizes determined (Table 1). The various patterns are shown in Figure 10 to Figure 14. HA12 was prepared from

two different preparations differing only slightly in their distributions.

The segments were measured in 0.5 M NaCl using dilute solution viscometry to determine the hydrodynamic molecular weights of the segments. Plots of reduced viscosity versus concentration are shown in Figure 15 and Figure 16. The intrinsic viscosity and slope of each plot (Table 2) was also determined by linear least squares analysis. From the slopes, the value of K was also determined using the Higgins relationship

$$\eta_{sp} / C = [\eta] + K' [\eta]^2 C$$

Eq. 12

The intrinsic viscosity values of the HA segment samples were used to calculate the apparent

molecular weight, using viscosity- molecular weight relationships devised by two other groups. Cleland^{37 40} examined low molecular weight HA samples at 25 degrees centigrade in 0.2M NaCl, and found the data to fit the equation:

$$[\eta] = 0.0028 M_w$$

Eq. 13

in which, M_w had been determined by light scattering or sedimentation equilibrium measurements. Shimada and Matsumura (28) similarly studied low molecular HA samples at 37 degrees centigrade in 0.2 M sodium phosphate buffer, pH 7.3, by Viscometry and sedimentation equilibrium. They determined the following equation:

10 % Polyacrylamide Gel Electrophoresis of Purified Oligosaccharides

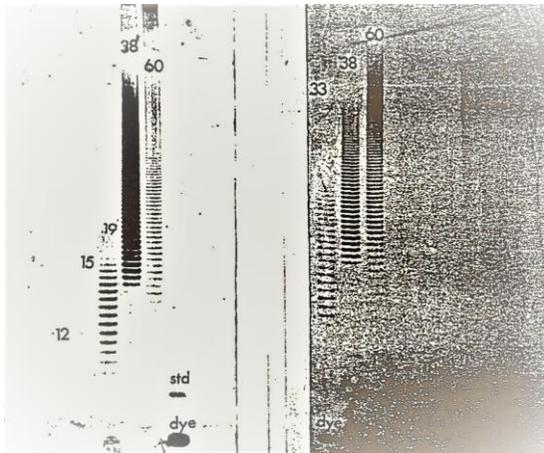


Figure 9. 10 % Polyacrylamide gel electrophoresis of purified oligosaccharides having different molecular weight distributions. Fractions were purified on a Bio-Gel P-60 column equilibrated with 0.5 M NaCl and separated on a 32 x 14 x 0.15 cm slab gel using a combined alcian blue and silver staining technique.^{63 111}

¹¹¹ Min, Hoki, and Mary K. Cowman. "Combined alcian blue and silver staining of glycosaminoglycans in polyacrylamide gels: application to electrophoretic analysis of molecular weight distribution." *Analytical biochemistry* 155, no. 2 (1986): 275-285.

HA Segment Sizes

Segment	Mw/#disacch.	Mn/#disacch.	Range
HA12	4590/12	4590/12	9-15
HA12	4970/12	4880/12	9-16
HA15	6180/15	6090/15	10-23
HA19	7830/20	7560/19	13-30
HA33	13200/33	11600/29	17-72
HA38	15100/38	14000/35	21-66
HA60	24200/60	15400/38	16-100

Table 1. Hyaluronic acid segment sizes as determined by gel electrophoretic scans. All segments are in 0.15 M NaCl.

Molecular weight distribution patterns for HA Segments (HA15 and HA12)

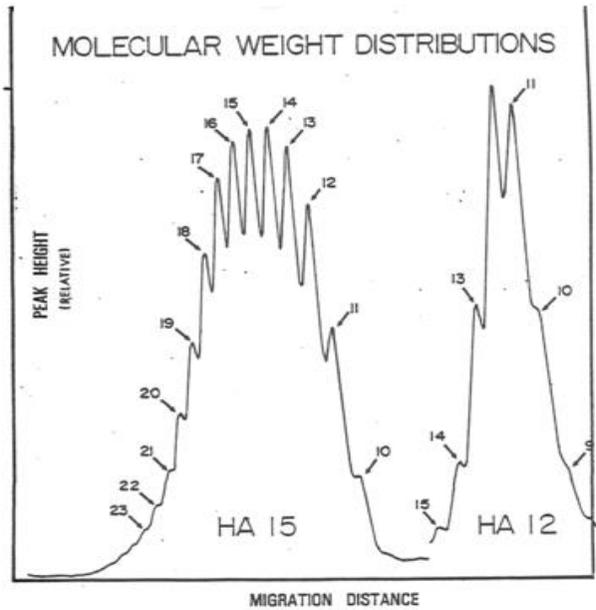


Figure 10. Molecular weight distribution patterns for HA segments. Gels were scanned at 615 nm and peak positions determined from standards of know molecular weights, which were co-electrophoresed with the segments. No correction was made for the chain length dependence of alcian blue staining previously discussed. The numbers in the figure represent the number of repeat units per oligosaccharide species.

Molecular Weight Distribution Patterns for HA Segments (HA12 and HA19)

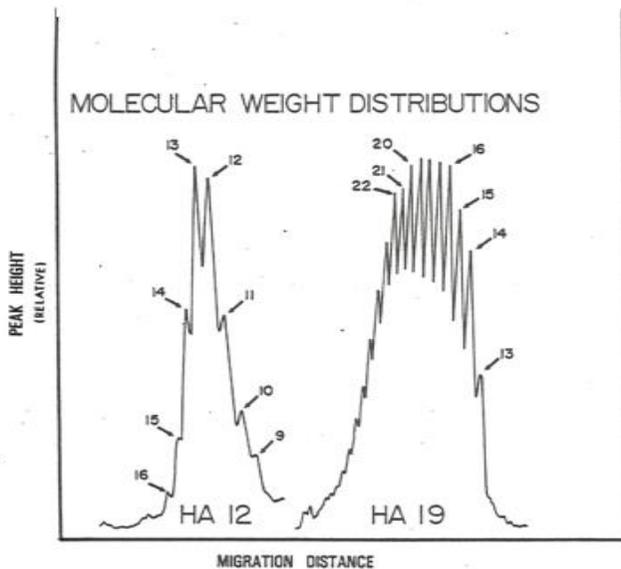


Figure 11. Molecular weight distribution patterns for HA segments. Gels were scanned at 615 nm and peak positions determined from standards of know molecular weights, which were co-electrophoresed with the segments. No correction was made for the chain length dependence of alcian blue staining previously discussed. The numbers in the figure represent the number of repeat units per oligosaccharide species.

Molecular Weight Distribution Patterns for HA Segments (HA33)

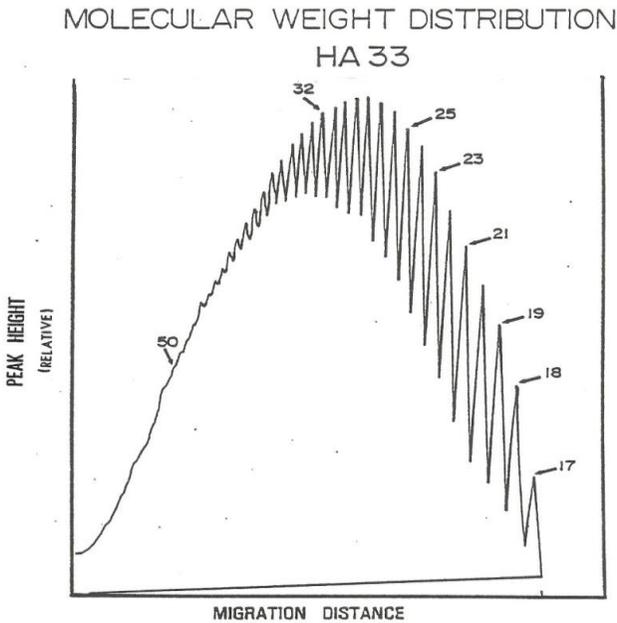


Figure 12. Molecular weight distribution patterns for HA33 segment. Gels were scanned at 615 nm and peak positions determined from standards of know molecular weights, which were co-electrophoresed with the segments. No correction was made for the chain length dependence of alcian blue staining previously discussed. The numbers in the figure represent the number of repeat units per oligosaccharide species.

Molecular Weight Distribution Patterns for HA Segments (HA38)

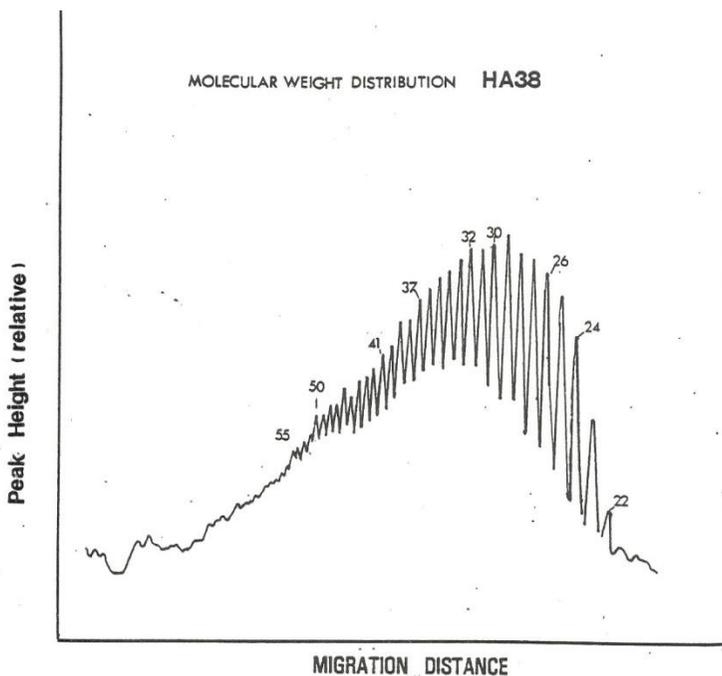


Figure 13. Molecular weight distribution patterns for HA38 segments. Gels were scanned at 615 nm and peak positions determined from standards of known molecular weights, which were co-electrophoresed with the segments. The numbers in the figure represent the number of repeat units per oligosaccharide species.

Molecular Weight Distribution Patterns for HA Segments (HA60)

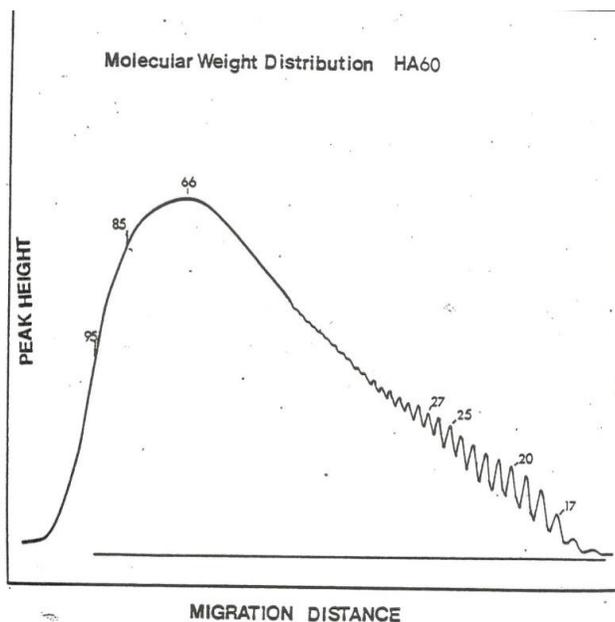


Figure 14. Molecular weight distribution pattern for HA60 segments. Gel was scanned at 615 nm and peak positions determined from standards of known molecular weight distributions, which were co-electrophoresed with the segment. Estimation of peak molecular weights, which were measured directly, was obtained by plotting log Mw versus gel migration distance and interpolating. The numbers in the figure represent the number of repeat units per oligosaccharide species.

Intrinsic Viscosity of HA Segments in 0.15 M NaCl (HA12 and HA15)

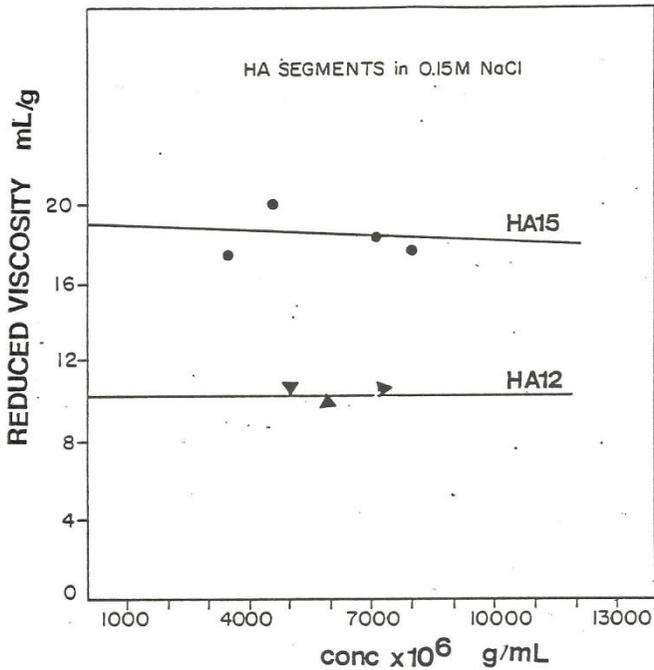


Figure 15. Intrinsic viscosity of HA segments in 0.15 M NaCl. All segments were pre-filtered through a 0.2-micron filter. Sample concentration was determined by the modified carbazole method.¹⁰³

Intrinsic Viscosity of HA Segments in 0.15 M NaCl (HA33 and HA38)

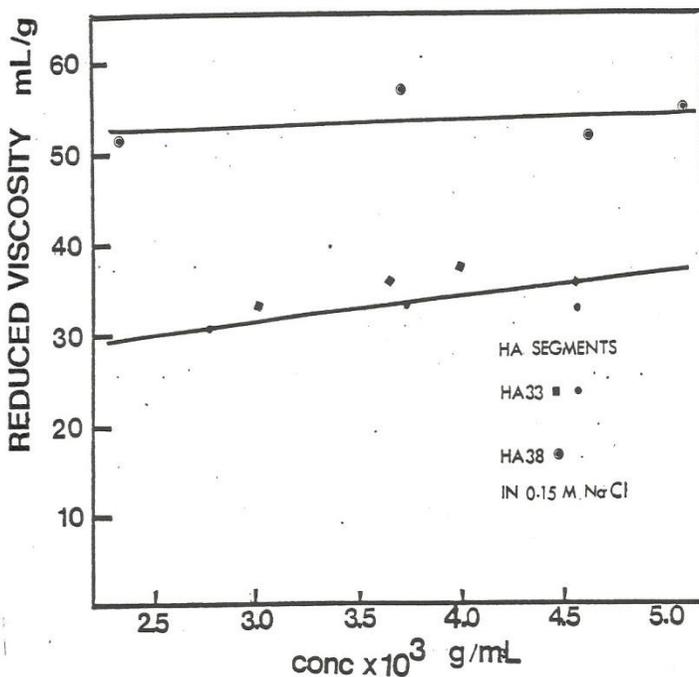


Figure 16. Intrinsic viscosity of HA segments in 0.15 M NaCl. All segments were pre-filtered through a 0.2-micron filter. Sample concentration was determined by the modified carbozole method.¹⁰³ Note that the position of the zero concentration on the abscissa is off-scale.

Viscometric Data
For HA Segments ^a

Sample	$[\eta]$	K'	Mw (PAGE)	Mw Cle ^b	Mw Shi ^c
HA12	10	-0.11	4590	3570	5880
HA15	19	-0.29	6180	6790	10000
HA33	29	0.23	13200	10400	14300
HA38	51	2.20	15100	18200	22800

Table 2. Viscometry data for HA segments in 0.15 M NaCl at 25 degrees centigrade ^a. Molecular weights are determined by polyacrylamide gel electrophoresis (PAGE) and compared with the results obtained by Cleland (Cle) ^b, $Mw = [\eta]/0.0028$; or Shimada and Matsumura (Shi) ^c, $Mw = ([\eta]/3.0 \times 10^{-4})^{1/1.2}$.

$$[\eta] = 3.0 \times 10^{-4} M^{1.2}$$

Eq. 14

The above relationship yields values for the apparent molecular weight of each HA segment sample in the present study, ignoring differences in Ionic strength and temperature. These apparent molecular weights may be compared

with the weight average molecular weight values determined by polyacrylamide gel electrophoresis. A moderately good agreement is found with the data of Cleland (plus or minus 20%), but less good agreement with the data of Shimada and Matsumura (our molecular weight is generally 10-50% lower than predicted) presumably because of the temperature dependence.²⁸

To better determine the viscosity - molecular weight relationship for the HA segments under our conditions, a plot of $\log M_w$ versus $\log [\eta]$ (figure 3.9) was made. The data were fit by a linear least squares analysis yielding the relationship:

$$[\eta] = 8.26 \times 10^{-4} M^{1.13}$$

Eq. 15

This relationship is in reasonable agreement with previous studies. The $M^{1.13}$ dependence indicates an extended coil.

The Huggins constant k' was generally found to be of low magnitude. The slightly negative values observed for HA12 and HA15 probably reflect experimental error and probably approach zero. For HA38, $k' = 0.23$. The transition from values of approximately 0 to 0.25 was previously observed by Shimada and Matsumura for HA segments of increasing size in the same molecular weight range. The high value of $k' = 2.2$ for H33 is unexplained at the present, but was reproduced in delicate trials. There is no evidence for substantial segment

segregation in 0.15 M NaCl in this work. The viscosity molecular weight relationship developed by Shimada and Matsumura (28) and Cleland,^{37 40} based on hydrodynamic data, are in reasonable agreement with our data, based on the electrophoretic analysis of molecular weight.

Characterization of Sodium Hyaluronate

Hyaluronic acid (Healon) was measured several times by dilute solution Viscometry as well as by light scattering. The intrinsic viscosity determined by least squares analysis was found to be 2900 ml/g with a standard deviation of 360 ml/g (

Table 3) and Figure 19. Using the relationship determined by Balazs⁷⁵ for high molecular

weight HA:

$$[\eta] = 0.029 M^{0.8} ;$$

Eq. 16

The molecular weight of our HA sample was calculated to be 1.8×10^6 . The k value was 0.76, which is somewhat larger than expected for a non-interacting random coil polymer in a good solvent.

Low Angle Laser Light Scattering (LALLS) measurements also gave a polymer molecular weight of 1.8×10^6 (Figure 19). The second virial coefficient, A_2 , was 2.7×10^{-3} ml-mole /g². This compares to a value of approximately 3×10^{-3} ml-mole /g² for a polymer molecular weight of 2×10^6 determined by Wik.⁸⁷

Mixtures of Polymer and Segment

Various ratios of polymer and segment were mixed and subsequently measured after 0.5 - 24 hours by Viscometry. Figure 20 and Figure 21 show the reduced viscosity plots obtained.

The intrinsic viscosity values measured may be used to calculate apparent average molecular weights of the mixtures (Table 4). For this calculation, the viscosity molecular weight relation of Balazs⁷⁵ for polymeric HA was employed. The degree to which this assumption is appropriate will be discussed below. The value so calculated is termed M_o in Table 4. The apparent molecular weight, M_o , may be compared to the calculated viscosity average molecular weight, M_o , based on the known polymer and segment molecular weights and

their ratio of mixing. For this calculation, the mixture was considered to be composed of two mono-dispersed species. The molecular weight of each being given by the weight average molecular weight of that component.

For HA plus HA12, HA15, or HA1, the ratio of the observed apparent average molecular weight (M_o) to expected molecular weight (M_c) is close to 1.0. Thus, no evidence for interaction between segments and polymer (e.g., attachment of segments to polymer, or disruption of polymer aggregates) was conserved on this basis. For HA38, a slightly higher molecular weight was observed than had calculated. This result prompted us to investigate larger HA segments by the same procedure (Fig. 3.16 and 3.17). One

sample of HA60 plus polymer gave a high apparent molecular weight, similar to the HA38 result. However, other samples gave values approximately in agreement with the expected molecular weight, or showed extremely low intrinsic viscosity and poor reproducibility. The causes of this variability are not known, but could relate to variable degrees of interaction between segments and polymer leading to metastable states.

The k' values of the mixtures show a significant variation as a function of ratio of mixing and segment chain length. For HA plus HA12, the k' value was 0.00 to 0.24. For HA plus HA15, considerably higher k' values (1.98, 3.25) were observed. These values may indicate

association of the segments with the polymer to some extent.

However, larger segments failed to show such high k' values. Indeed, some samples (HA38 and HA60) gave the opposite result (i.e., very low k' values). It is possible that conformational changes, accompanying interaction of polymer plus segments, contributes this unexpected result.

A composite graph of the data for the mixtures is given in Figure 22. These dates are for those mixtures with nearly equal weight ratios of segments and polymer. It is clear that the slopes may be either higher or lower than expected. An alternative view of these data may be obtained by calculating and plotting the data

based on the polymeric HA (Healon) concentration alone. In this view (Figure 23), the segments are considered purely as a perturbant, whose contribution to the solution viscosity at the low concentration employed is negligible. Only HA38 and HA60 mixtures with the polymer show evidence of an increase in the intrinsic viscosity and therefore apparent molecular weight. These data are suggestive of some degree of interaction between polymer and segments in 0.15M NaCl provided the segments be of sufficient length.

Discussion

Isolated Segments

No evidence of chain association is found for

isolated segments in 0.15M NaCl. The results of this work lead to a reasonable relationship between the intrinsic viscosities and molecular weights for low molecular weight oligosaccharides. Electrophoresis can be used to estimate the molecular weights of low molecular weight oligosaccharides in 0.15M NaCl.

The Importance of Chain Length in HA Network Formation

The viscosity results for the mixtures of polymeric HA with HA segments are highly indicative of intermolecular interaction.

The degree and type of interaction appears to depend on the chain length of the segments employed and possibly on the weight ratio of

polymer to segments. With the longest segments studied (HA38 and HA60), some samples showed a higher intrinsic viscosity than expected. This observation may indicate association of the polymer and segments. The smaller segments (HA12, HA15, and HA19) did not cause the same effect. Thus a stable polymer - segment interaction may require a substantial segment length.

In addition to the intrinsic viscosity, the slopes of each reduced viscosity versus concentration plot were determined. The Huggins constant, k' , determined from the slope, is sensitive to polymer conformation, solvent, interactions, and association phenomena. We

found rather extreme variability in this parameter for the mixtures studied. Plausible explanations for the variability include the formation of either "weak" cross-links between polymers and segments (increase in k'), or disruption of polymer aggregates, conformational change, or change in solvation caused by the segments (decrease in k').

Further experiments will be required to elucidate the dependence of k' on experimental conditions (e.g., incubation period prior to analysis, method of mixing etc.).

A Matrix Requirement for Chain - Chain Association

This work indicates that chain-chain association may occur for higher oligomers in 0.15M NaCl, but only in the presence of high polymer. No evidence for the association of isolated oligomers was found. The interacting chain with the required minimum chain length may interact with segments of the polymer. The stoichiometry is not clear, however. The interaction appears to involve such factors as age of polymer - segment mixture and ratio of segment to polymer concentration.

It is conceivable that the polymer is capable of forming micro-domains of network structures. These domains may differ in average conformation from non-interacting polymer

segments or isolated segments. A stable association of segments with the polymer may be proposed to occur only at the sites of interaction within the polymer matrix. Just as with enzyme - substrate complexes, the segment - polymer interaction might require three-dimensional order for the complex, creating a local environment favoring segment binding. Thus in the absence of such order, (isolated segments), no such binding can occur.

Since there are optimal conditions for substrate binding in enzymes substrate complexes, which depend on such factors as pH, metal ions, and temperature, there may be optimal conditions for the binding of isolated segments to polymer or for the

binding of local regions of the polymers to itself. These processes are expected to be dynamic. The polymer can be viewed as a rapidly changing network where at any instant in time only interacting segments of the chain can serve as the substrate.

That hyaluronate can exist in an extended or a contracted form is supported by x-ray diffraction work on hydrated films ⁴⁵. It was further suggested that stiff chain segments could be accounted for by the formation of helical loops⁴⁴. Thus, conformationally ordered (single or double helix) sections of the polymer may provide the binding site for HA segments.

Viscosity of High Mol. Weight HA

Exper No.	Conc μg/ml	$[\eta]/C$	k'	$[\eta]$	$M_w(\text{obs})$
1	414	4120	0.46	2700	1.6
	248	3400			
	182	3390			
2	368	5630	0.32	3750	2.5
	231	4290			
	137	4670			
3	602	7740	0.79	3060	1.9
	370	5460			
	306	5660			
	296	4481			
	190	4962			
4	397	5640	0.72	2890	1.8
	300	4100			
	172	4300			
	139	3380			
	85	3650			
	62	3230			
5	405	5830	0.74	3080	1.9
	256	4920			
	196	5100			
	189	3920			
	128	3830			

$M_w = 1.8 \times 10^6$, $[\eta] = 2900 \pm 350 \text{ ml/g}$ and $k' = 0.79$

Table 3. Various samples of Healon EAB-1 were subjected to intrinsic viscosity methods in 0.15M NaCl after pre-filtering with a 0.45-micron filter. Five such measurements were used to determine the intrinsic viscosity of the polymer. Least square analysis was used to fit these data. K' was determined from the slope of η_{sp}/C versus concentration, where the slope is considered to be equal to $k' [\eta]^2$.

Relationship Between Mw and Intrinsic Viscosity

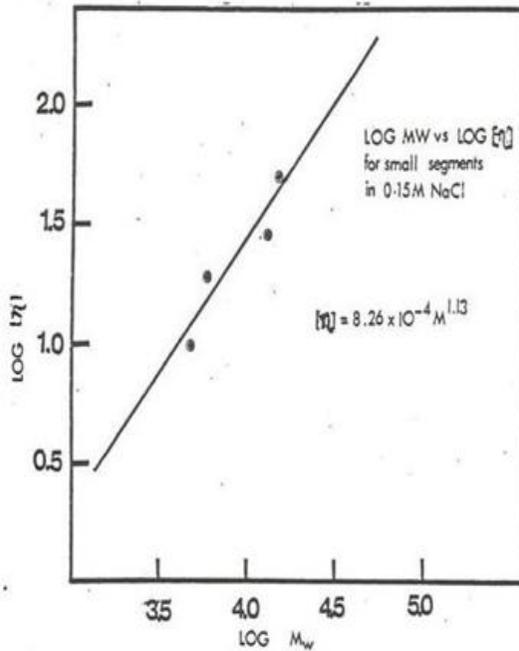


Figure 17. Relationship between M_w and intrinsic viscosity for all segments studied. Segments were all in 0.15 M NaCl.

Determination of Healon HA (EAB-1)
 Intrinsic Viscosity in 0.15M NaCl

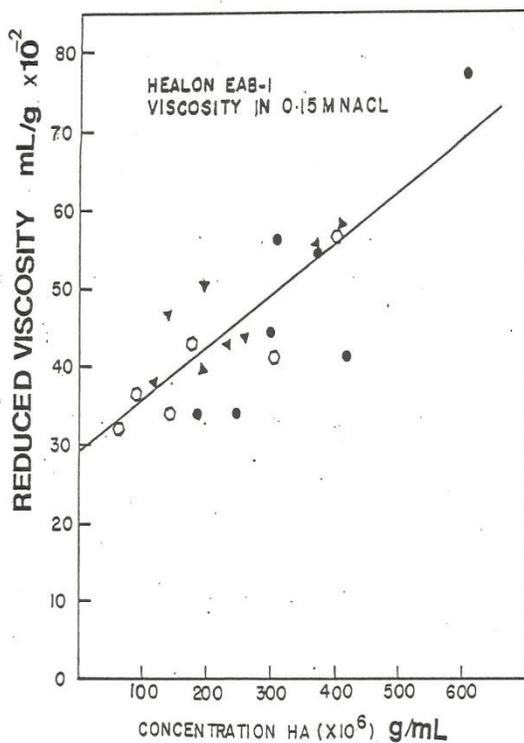


Figure 18. Determination of Healon HA (EAB-1) intrinsic viscosity in 0.15M NaCl. The results shown are from five determinations.

KMX-6 Low Angle Light Scattering of Healon HA (EAB-1) in 0.15M NaCl

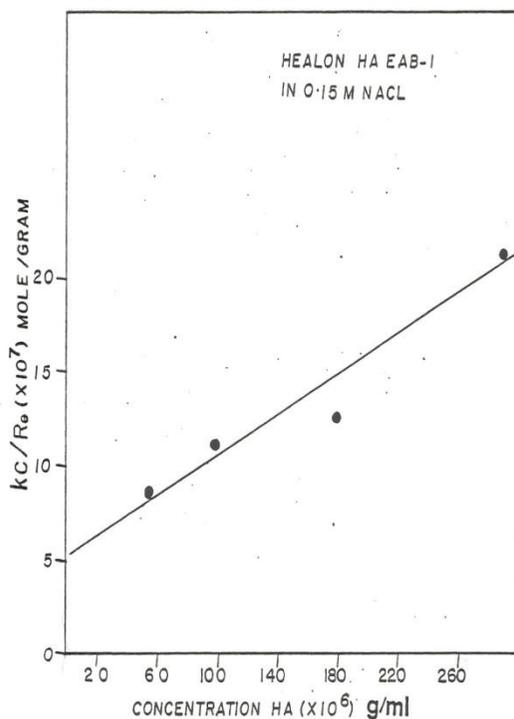


Figure 19. KMX-6 low angle light scattering of Healon HA (EAB-1) in 0.15M NaCl. Sample was filtered through a 0.45 μ m filter directly into flow cell. The automated carbazole method (100) was used to determine sample concentrations .

Mixture of HA Segments and HA Polymer in 0.15M NaCl (HA15 and HA12)

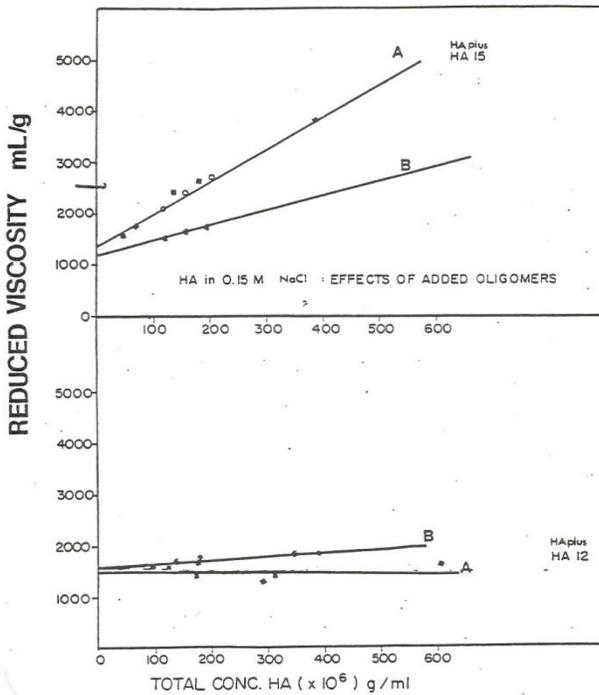


Figure 20. Mixture of HA segments and HA polymer in 0.15M NaCl. Measurements were made at various times and at various ratios of segment to polymer. A and B represent different preparations (see materials and methods). Measurements are plotted as a function of total HA in the mixture.

Mixture of HA Segments and HA Polymer in 0.15M NaCl (HA39 and HA19)

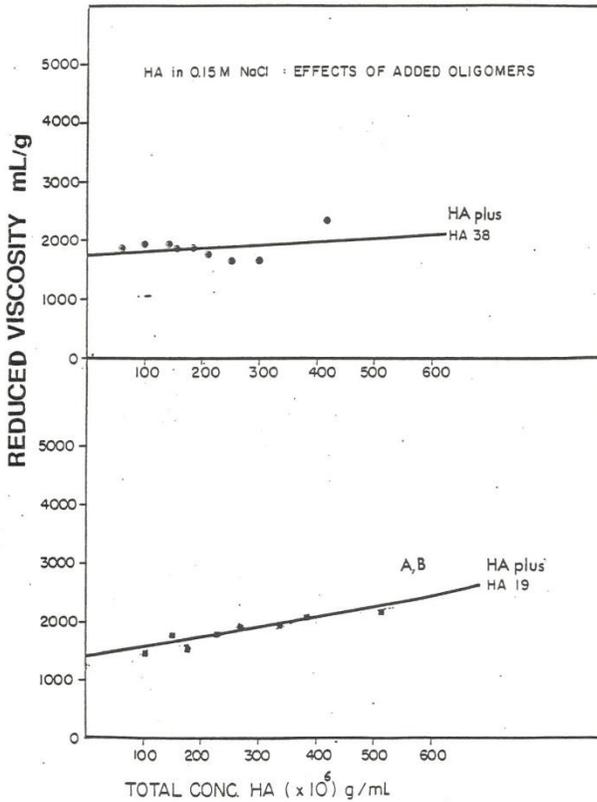


Figure 21. Mixture of HA segments and HA polymer in 0.15M NaCl. Measurements were made at various times and at various ratios of segment to polymer. Measurements are plotted as a function of total HA in the mixture.

Mixture of HA Segments and HA Polymer in 0.15M NaCl: Effects of Added Oligomers

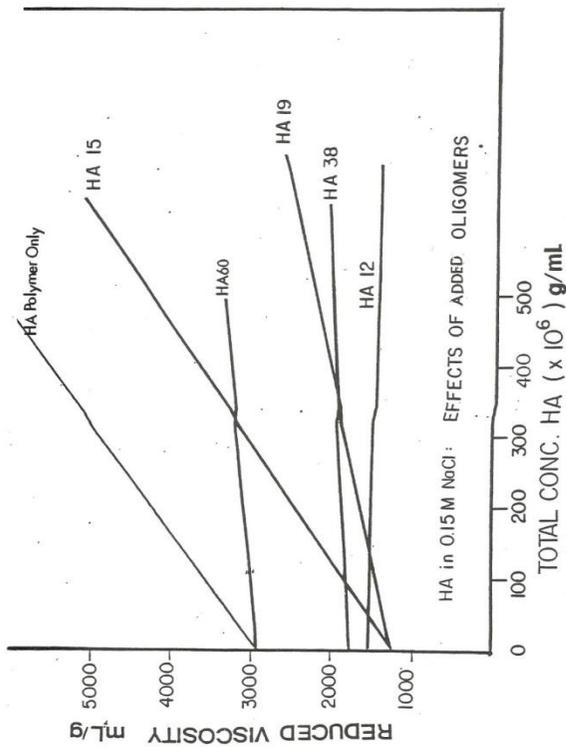


Figure 22. Mixture of HA segments and HA polymer in 0.15M NaCl. Measurements were made at various times and at various ratios of segment to polymer plotted as a function of total HA in the mixture.

Mixture of HA Segments and HA Polymer
in 0.15M NaCl: Effects of Added
Oligomers

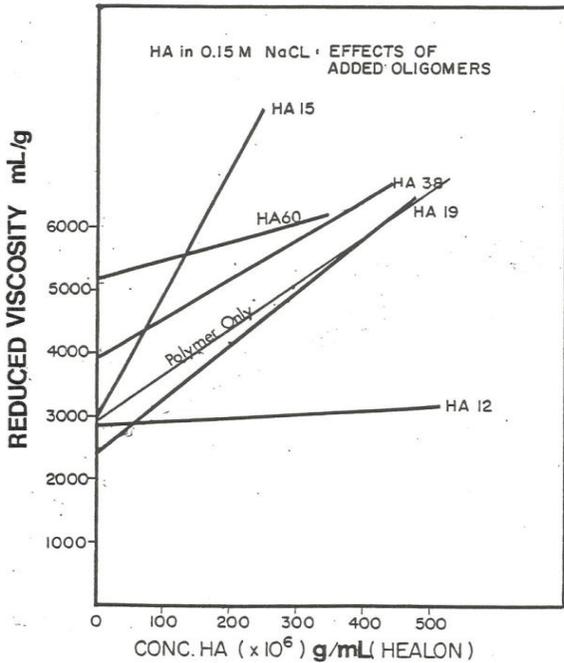


Figure 23. Mixture of HA segments and HA polymer in 0.15M NaCl. Measurements were made at various times and at various ratios of segment to polymer. A and B represent different preparations (see materials and methods). Measurements are plotted as a function of Healon HA concentration in the mixture.

Mixture of HA60 and HA Polymer in 0.15M NaCl

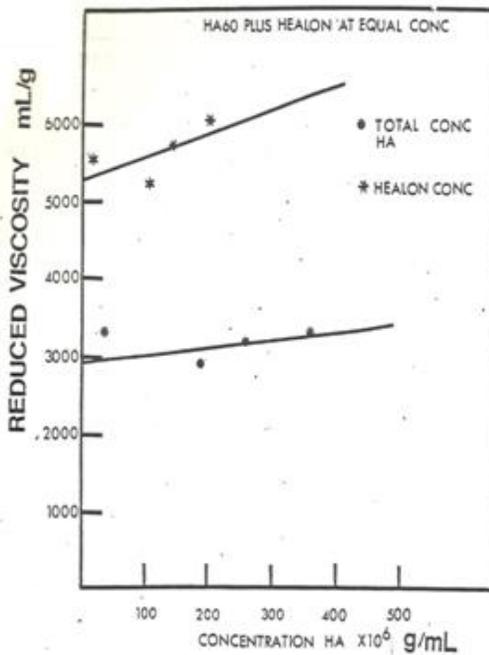


Figure 24. Mixture of HA60 and HA polymer in 0.15M NaCl. Measurements are made at nearly equal concentrations of segment and polymer. Measurements are plotted as a function of Healon HA concentration only and as a function of total concentration of HA in the mixture.

Mixture of HA60 and HA Polymer in 0.15M NaCl

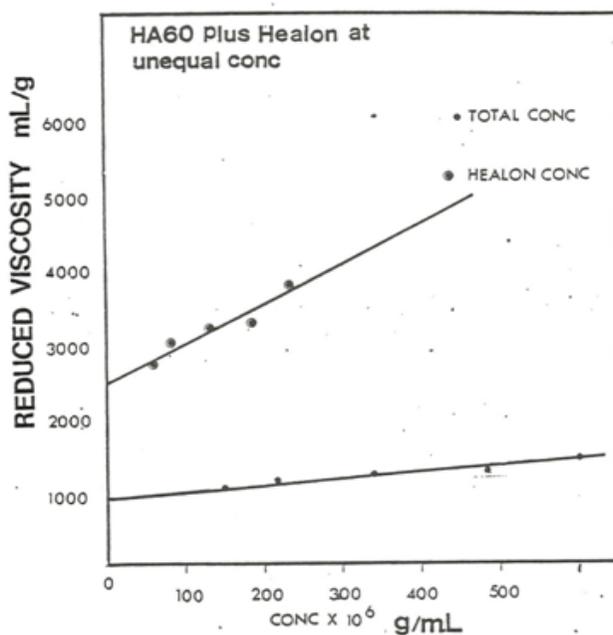


Figure 25. Mixture of HA60 and HA polymer in 0.15M NaCl. Measurements are made at unequal concentrations of segment and polymer. Measurements are plotted as a function of Healon HA concentration only and as a function of total concentration of HA in the mixture.

Polymer Plus Segment Data

Type Seg.	S/P Ratio	$[\eta]_0$ ml/g	$[\eta]_c$	$[M_o]$ g/mo l $\times 10^{-5}$	M_c	K_b	K'_b/k'_p $k'_p=0$.79	M_o/M_c
HA 12	1.00 0.67	150 0 160 0	1440 1760	7.8 8.4	7.4 9.5	0.00 0.24	0.0 0.3	1.0 0.9
HA 15	0.88 1.80	140 0 121 0	1570 1120	7.1 5.9	8.3 5.4	3.25 1.98	4.1 2.5	0.9 1.0
HA 19	1.00 0.96	157 0 126 0	1470 1500	8.2 6.3	7.6 7.8	0.47 1.27	0.6 1.6	1.1 0.8
HA 38	1.29	176 0	1320	9.5	6.6	0.19	0.2	1.4
HA 60	1.60 0.80	----- 290 0	1170 1660	4.5 18.0	5.6 8.9	0.82 0.12	1.0 0.2	0.8 2.0

Table 4. Data from hyaluronic acid plus HA segments. All measurements were in 0.15M NaCl. k' was calculated from the slope of η_{sp}/C versus concentration data (total concentration in mixture). M_o was the observed molecular weight while M_c was the calculated molecular weight. k'_p represents polymer and k'_0 represents the mixture of polymer plus segment.

Chapter 4. Solvent and Chain Length Effect on the Circular Dichroism of Sodium Hyaluronate Segments.

Introduction

HA is a high molecular weight biopolymer found in connective tissue. It is composed of alternating N-acetyl- β -D-glucosamine and β -D-glucuronic acid residues linked at the beta 1, 3 and the β - 1, 4 positions respectively.⁸⁹ The polymer has previously described as a random coil polyelectrolyte with some rigidity.^{85 90} Inter-residue hydrogen bonding has been suggested,^{22 34 94-97} but in aqueous solution hydrogen bonding to solvent water might predominate.²³ Various studies on HA and HA oligomers involving Viscometry and chiroptical methods ³⁹⁻

^{41 54 55 90 91} appear to provide evidence for chain-chain association leading to network formation in the polymer.

Recently, cationic dye binding experiments appear to provide evidence for chain-chain association or at least a transition from interacting to non-interacting species for very small segments.⁸⁸ The requirement for chain-chain association for small oligomers might not be the same as the requirement for high polymer. For one thing, in the polymer, intramolecular association might complicate intermolecular association, which might be more important than is the case of small oligomers. With regard to the polymer, there may be a chain length dependent Conformational

transition from the unassociated to the associated form within a polymer chain as well as between two or more chains of a "making and breaking" nature. These interacting segments could be characterized by microscopic equilibrium constants in a complex way. Rees et al ⁷⁰ found that HA segments of approximately 60 disaccharides could uncouple polymeric HA and alter the rheological of the polymer to those resembling isolated chains. Chain segments less than four disaccharides long did not show this effect.

In this study we prepare a HA segments of different average chain lengths (between 12 and 60 repeat units and record their CD spectra under different solvent conditions and at

different concentrations. We wish to determine if a cooperative chain length dependent conformational transition exists within these size ranges and identify a minimum chain length capable of competitively inhibiting the network structure of the polymer.

Materials and Methods

Preparation and molecular weight analysis of HA segments are described in chapter three. For spectroscopic study, HA segment samples were dissolved in 0.15M NaCl. When solvent perturbation experiments were to be performed, the sample in 0.15M NaCl was mixed with 0.1M HCl to reach pH 2.5, and ethanol was added to a final concentration of 17%.

Circular dichroism spectra were recorded on a Jasco Model J500A spectropolarimeter, using cylindrical quartz optical cells of known path length (10^{-3} - 0.05 cm). Spectra were recorded at room temperature at a sensitivity of 0.2- 2.0 mdeg/cm. The spectral bandwidth was 1.0 nm. Pen time constant and scan rate were adjusted to allow at least eight pen responses per unit of spectral bandwidth. The instrument was routinely calibrated with d-10-camphorsulphonic acid. The total absorbance of sample solvent, and cell combination was limited to a maximum of 1.5 at the lowest wavelength studied. Molar ellipticity values, in units of $\text{deg cm}^2\text{dmol}^{-1}$, were calculated based on the

disaccharide residue concentration, using the measured uronic acid concentration. Signal to rms noise ratios were approximately 25:1 and 40:1 at 210 nm and 6:1 and 20:1 at 195-200 nm depending on concentration.

Results

Characterization of Sodium Hyaluronate

Healon HA was measured over a range of concentrations in 0.15 M NaCl (salt) and in SEA (0.15 M NaCl, PH 2.5, 17% ethanol solvent). See Figure 26. Average values for the molar ellipticities are shown in tables 4.3 and 4.4. A 0.05 cm quartz cell was used for all measurements. In salt, a peak maximum appears at 208 nm with a molar ellipticity of -10000 deg cm²dmol⁻¹. These results are for an

average of five measurements over a concentration range of 110 to 300 $\mu\text{g/ml}$ glucuronic acid.

The samples were reduced to pH 2.5 with HCl and made 17% ethanol with absolute ethanol. This solvent caused the polymer to undergo drastic spectral changes. A positive peak appears at 225nm with a cross over point at 218 nm. Below the cross over point, the negative molar ellipticity increases continuously to -23000 $\text{deg cm}^2\text{dmol}^{-1}$ and to a new maximum below 195 nm (Figure 26).

The spectra presented here in 0.15M NaCl or in SEA resemble those observed by Park and Charkrabarti.⁸² Several solvent related spectral

changes are discernable. A peak maximum between 208 and 209 with a molar ellipticity of -9500 was found for the polymer in neutral aqueous solution. Cowman et al⁵³ found a singular negative band below 220 nm equal to -10200 deg cm²dmol⁻¹.

Charkrabarti studied solvent induced changes in the polymer⁸² by dialyzing the sample against a 0.1 M NaCl solution containing the appropriate solvent. This group also observed a positive band at 225 nm of approximately +1000 deg cm²dmol⁻¹ under similar solvent conditions. The large negative ellipticity seen below 200 nm was also observed. It was suggested that these changes might be due to hydrogen bonding involving the carboxylate group since the effects

can be reversed with the addition of HCONH_2 or by neutralization.

Monomer and Octasaccharide Spectra

Spectra of GlcUA and GlcNAc (Figure 27 and table 4.1 and 4.2) were taken in aqueous salt solution and in mixed solvent (SEA). Figure 27 shows that GlcUA in 0.5M NaCl gives a spectrum with cross over points at 196 nm and 205 nm. The spectrum is low intensity. Upon acidification and ethanol addition, a single crossover point occurs at 224 nm with a positive peak of low intensity centered on 206 nm. GlcNAc in salt gives a maximum at $-4900 \text{ deg cm}^2\text{dmol}^{-1}$ centered at 208 nm. This peak is reduced to $-2400 \text{ deg cm}^2\text{dmol}^{-1}$ in SEA. The

summed contributions of the two monomers at 210 nm equal $-5151 \text{ deg cm}^2\text{dmol}^{-1}$. This agrees quite well with the results of Cowman et al⁵³ of $-4700 \text{ deg cm}^2\text{dmol}^{-1}$ in neutral aqueous solution.

Buffington et al⁸³ examined the far UV CD of N-acetylglucosamine, glucosamine, glucuronic acid, and hyaluronic acid. $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions were assigned to amide and carboxylate chromophore. A non-additive contribution to the CD spectra was observed in the polymer relative to the monomer units. Monomer spectra in salt were in reasonable agreement with the results of Cowman et al⁷⁹ and Buffington et al⁸³ except that the molar ellipticity of GlcUA was somewhat reduced in intensity in the present case. However, the peak

centered on 210 nm with a molar ellipticity of -5000 deg cm²dmol⁻¹ for GlcNAc (fig. 4.2), is in agreement with the reported value at that wavelength.^{79 83}

HA4 (GlcUA-GlcNAc)₄ was measured to determine if any spectral changes occurred in either in 0.15 M NaCl or SEA for low molecular weight oligomers (Figure 28). A maximum molar ellipticity of -9570 deg cm²dmol⁻¹ appears at 208 nm. This peak is red shifted to 210 nm and reduced in intensity to -6170 deg cm²dmol⁻¹ at 208 nm in SEA. The octasaccharide spectrum in salt was in agreement with that obtained by Cowman et al.⁵³ The authors found a molar ellipticity of approximately -9000 deg cm²dmol⁻¹ at 210 nm in neutral aqueous solution. The

spectral changes for HA4 in SEA are similar in nature to the changes observed for the monosaccharide components.

The results obtained for monomers, octasaccharide, and hyaluronic acid in 0.15 M NaCl and in SEA are in reasonable agreement with the literature despite the fact that most of the reported spectra were taken in neutral aqueous solution in the absence of salt.

HA Segments

HA oligosaccharides were produced as previously described (chapter 3) and spectral properties investigated in 0.15M NaCl (fig. 4.4 and table 4.3) and in SEA (fig. 4.5 and table 4.4). The samples in 0.15 M NaCl gave spectra

resembling that of Healon HA (

Figure 26). For Healon HA, the average molar ellipticity is $-10000 \text{ deg cm}^2\text{dmol}^{-1}$ at 208 nm and the average molar ellipticity for all the segments studied is $-10170 \text{ deg cm}^2\text{dmol}^{-1}$ at 208 nm (Figure 29 and table 4.3).

When the segments are studied in SEA solvent, drastic spectra changes are seen. HA12 and HA19 show a similar spectra shape to HA4 in SEA solvent. Variations in the magnitude of the negative band are attributed to difficulties in sample concentration measurements. HA38 and HA60 show an altered spectra appearance. Below 200 nm, evidence of a negative band contribution remains much less than that of the polymer.

Park and Charkrabarti¹¹² found that the CD spectral change for low molecular weight HA was much smaller than that for the polymer in perturbing solvent and they suggest that the new conformation for hyaluronic acid is molecular weight dependent, solvent concentration dependent, pH, and temperature dependent. It was further noted that the carboxylate group was involved in the conformational transition of hyaluronic acid. This latter conclusion was based on work done

¹¹² Park, Joon Woo, and Bireswar Chakrabarti. "Conformational transition of hyaluronic acid carboxylic group participation and thermal effect." *Biochimica et Biophysica Acta (BBA)-General Subjects* 541, no. 2 (1978): 263-269.

on low molecular weight methyl hyaluronate of 30 ml/gm intrinsic viscosity.¹¹² The changes they saw were proposed to be due to increased intermolecular association of polymer segments, which are molecular weight dependent.

Concentration Dependence

HA33 was prepared and characterized as previously described (chapter 3). The sample was studied at various concentrations in a CD cell of varying path lengths (Figure 31). Although variation in peak intensity can be seen for various samples in salt solution, the average molar ellipticity of $-1000 \text{ deg cm}^2\text{dmol}^{-1}$ is in good agreement with the results obtained for octasaccharide and polymer. However, reduction

of solution pH to 2.5 and reduction in solvent polarity with the addition of 17% ethanol, leads to concentration dependent spectral changes below 200 nm (Figure 32). Below 200 nm, the negative molar ellipticity increases in intensity as the concentration is raised and eventually begins to resemble the spectrum seen for the polymer in SEA (Figure 26). A plot of the molar ellipticities at 196 nm versus molar concentration (Figure 33) demonstrates more clearly the effects of HA33 concentration.

In order to investigate the basis of the concentration dependence, an attempt was

made to fit the CD data by a two state aggregation equilibrium model.¹¹³ HA segments are considered to be either unassociated (monomeric) species designated M, or aggregates containing x segments, designated M_x. The equilibrium between these species is given by the expression:

$$xM = M_x$$

Eq. 17

for which the equilibrium constant may be expressed as:

¹¹³ Eisenberg, D., D. Crothers, and M. Donald. "Chemical and biochemical kinetics." *Physical Chemistry with Applications to the Life Sciences*, Benjamin/Cummings, Menlo Park, CA (1979): 572-573.

$$K = [M_x] / [M]^x$$

Eq. 18

The above equation may be rearranged to yield:

$$\log (x [M_x]) = x \log [M] + \log K + \log x$$

Eq. 19

We wish to determine the value of x by plotting $\log (x [M_x])$ versus $\log [M]$. The values of $[M]$, the concentration of unassociated chains, can be determined by assigning molar ellipticity values for the completely dissociated and the completely associated states. The dissociated state is considered to be well modeled by the value of $[\theta]_{196}$ for low molecular weight HA segments in SEA, for which $[\theta]_{196} \approx -20000 \text{ deg cm}^2\text{dmol}^{-1}$. (This assumption is almost undoubtedly false since it is unlikely that the

polymer is completely aggregated under our conditions). Using the assigned values for the two states as given above, the fraction of HA33 segments which is present in solution as monomeric species may be expressed as:

$$f = 1 - \frac{[\theta] - [\theta] M}{[\theta] M_x - [\theta] M}$$

Eq. 20

where $[\theta]$ is the observed value at a given concentration. The total concentration of the monomeric species is $f_M [M]_T$, where $[M]_T$ is the total concentration of the HA segments originally present in solution.

The value of $x [M_x]$ is simple expressed by considering that the total concentration is the sum of the monomer species concentration and x times the concentration of aggregate.

Therefore:

$$x [M_x] = [M]_T - [M]$$

Eq. 21

The data for a series of four HA33 samples are given in table 4.5. It is plotted in Figure 34. Using all available data points, the least squares fit gives a slope of 3.2. If the lowest concentration data point is eliminated based on its disagreement with similar studies on HA38, the slope is calculated to be 1.7. Thus the data could be compatible with either dimerization ($x=2$) or trimerization ($x=3$).

These results indicate that over the concentration range studied, HA33 is capable of intermolecular association in perturbing solvent.

HA Polymer Plus Segment

Based on our data for HA segments in SEA, association of the segments is shown to occur even in the absence of polymer. However, the strong interaction of polymer with segments compared to segments alone (as observed by viscometry in 0.15M NaCl), led us to investigate the CD properties of polymer plus segment mixtures in SEA and 0.15 M NaCl.

The CD spectra of mixtures containing HA60 and HA polymer are given in Figure 35. These spectra were compared with the spectra calculated based on no interaction between polymer and segment (i.e., the weighed average of the polymer and segment spectra in the appropriate solvent). The difference spectra are given in Figure 36.

It is not completely certain that appreciable association of polymer and segment occurs in 0.15M NaCl, but a dramatic non-additive

contribution to the CD spectra is seen for the mixture in SEA. This difference is not attributed to the effective doubling of the segment concentration via mixing with the polymer, since in the concentration range used there is little significant change in the spectrum of HA60.

HA octasaccharide was also added to polymer to see if any contribution for very low molecular weight oligosaccharides could occur. At a higher ratio of segment to polymer than used for HA60, no significant contribution to the difference spectrum of the mixture could be discerned (Figure 37) in either salt of SEA. The evidence indicates that HA60 and polymer are capable of aggregation in mixed solvent and maybe even in salt. HA4 does not interact with polymer. These

data are in agreement with the viscosity studies.

*Discussion:
Chain Length Dependence of HA Segment
Aggregation*

These data indicate that in 0.15M NaCl, isolated segments of sodium hyaluronate up to 60 disaccharides in length are not capable of chain association. However, perturbing solvent conditions can lead to spectral changes indicative of increased intermolecular association or aggregation. It is proposed that there exists a minimum chain length requirement for chain association. In the present work which was done at low polymer and segment concentrations (200 μ g/ml GlcUA or less), this minimum size range appears to be

between 33 and 38 disaccharides in length (see Figure 30 and Figure 32). Because the aggregation is concentration dependent, increasing the concentration of segment may also decrease the chain length requirement for association. If the on-set of polymer-like behavior occurs at a degree of polymerization many times less than polymer, then these segments could replace polymer in certain important applications.

Concentration Dependence of Chain-Chain Association

HA33 was studied at various concentrations and at various cell path lengths in salt (fig.4.6) and in SEA (fig. 4.7). It appears that HA33 aggregation is concentration dependent over the

concentration range studied (2 to 12 mM GlcUA). An average degree of aggregation of 2.5 was found (Figure 34). Increasing the concentration of the segment increases intermolecular association leading to aggregation.

Stakus and Johnson¹¹⁴ (personal communication) worked at 0.1-10 mM concentration of repeating disaccharides in 45% ethanol at PH 2.5. The group found evidence of intermolecular association, well fit by a

114 Stakus, P. W., and W. C. Johnson. "A Double Stranded Secondary Structure for hyaluronic Acid in Organic Solvent as Revealed by Vacuum UV Circular Dichroism." In *Biophysical Journal* vol. 49, no. 2, pp. A336-A336. 9650 Rockville Pike, Bethesda, MD 20814-3998 Biophysical Society, 1986.

dimerization model, beginning at eight repeat units. Oligomers ranging from 12 to 16 disaccharides in length gave concentration dependent spectra primarily above 1 mM concentration. These results should be compared with the results of this work. Working at low ethanol concentration and at a sample concentration less than 2 mM disaccharide and in 0.15 M NaCl, we found evidence of a chain length dependence of CD spectra (at 196 nm) beginning at a chain length between 33 and 38 disaccharides. Our data were also consistent with dimerization, within experimental error.

Although the minimum size for association appears to depend on solvent conditions and concentration, it is clear that the association of

sodium hyaluronate is extremely molecular weight and concentration dependent.

Polymer Plus Segment

The addition of HA60 to polymer leads to a non-additive spectral contribution in 0.15MNaCl and SEA. This effect is not observed for octasaccharide. The results seen in 0.15 M NaCl are less dramatic than those seen in SEA, but do suggest that the association or the binding of segment to polymer in salt solution can occur to some extent. In SEA, the equilibrium is shifted in favor of aggregation and a large non-additive contribution to the spectrum is seen. It is reasonable that HA60 should aggregate with the polymer since HA33 can aggregate with itself

Welsh et al⁶⁷ report that segments of approximately 60 disaccharides can uncouple the polymer network structure of hyaluronic acid thereby reducing its rheological properties to those resembling isolated chains with no evidence of coupling. The non-additive contribution to the CD spectra for the mixture is attributed to the strong interaction of HA60 with the polymer, leading to an increased degree of association.

CD spectra of hyaluronic acid in salt and in mixed solvent (SEA)

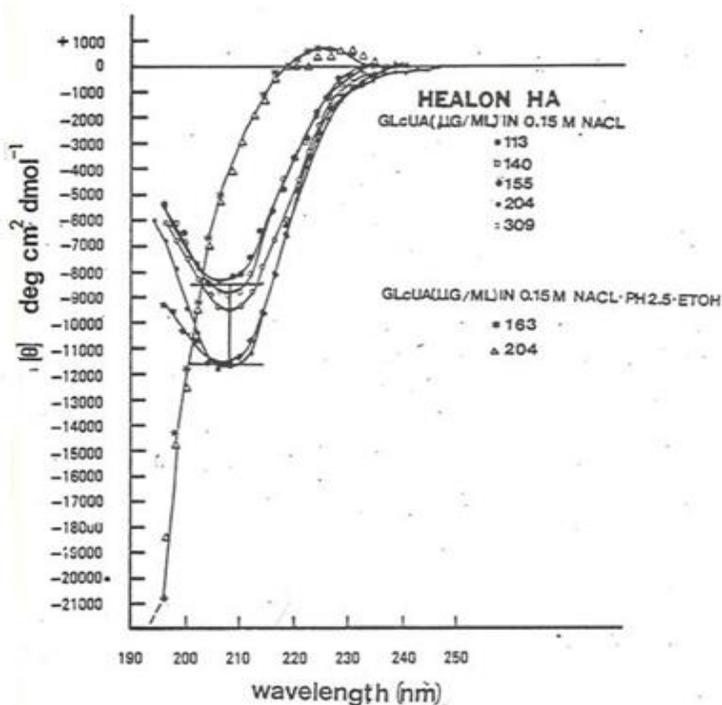


Figure 26. CD spectra of hyaluronic acid in salt and in mixed solvent (SEA). The sample cell was 0.05 cm. Sample was first measured in salt, converted to SEA solvent, left overnight and rescanned. Figure shows the molar ellipticity of the polymer as a function of wavelength at low concentration.

CD Molar Ellipticity of Small HA Segments and Monomers in 0.15M NaCl

NM.	GlcUA	GlcNAc	HA4	HA4(2)
194	-460	0		
196	0	-7380	-6260	-8620
198	+150	-2030	-6880	-8380
200	+270	-2770	-7420	-8260
202	+250	-3820	8050	-8680
204	+80	-4280	-8670	-9520
206	-60	-4740	-8850	-9700
208	-230	-4880	-9210	-9940
210	-370	-4790	-8760	-9760
212	-430	-4550	-7960	-8920
214	-450	-4140	-7240	-7840
216	-420	-3590	-6350	-7010
218	-370	-2990	-5190	-5390
220	-290	-2480	-3840	-4550
222	-220	-1980	-2950	-3230
224	-170	-1470	-2320	-2460
226	-110	-920	-1610	-1620
228	-80	-690	-1070	-1020
230	-50	-460	-540	-720
232	-10	-280	-270	-360
234	0	-180	0	-180
236	0	-50	0	0
238	0	0	0	0
240	0	0	0	0
242	0	0	0	0
244	0	0	0	0
256	0	0	0	0

Table 5. Monomer and oligosaccharide spectral data in 0.15MNaCl. Molar Ellipticity is listed as a function of wavelength. HA4 was measured at 217 and 324 (2) µg/ml GlcUA in a 0.05 cm cell

HA Monomer Spectra as a Function of Solvent Type

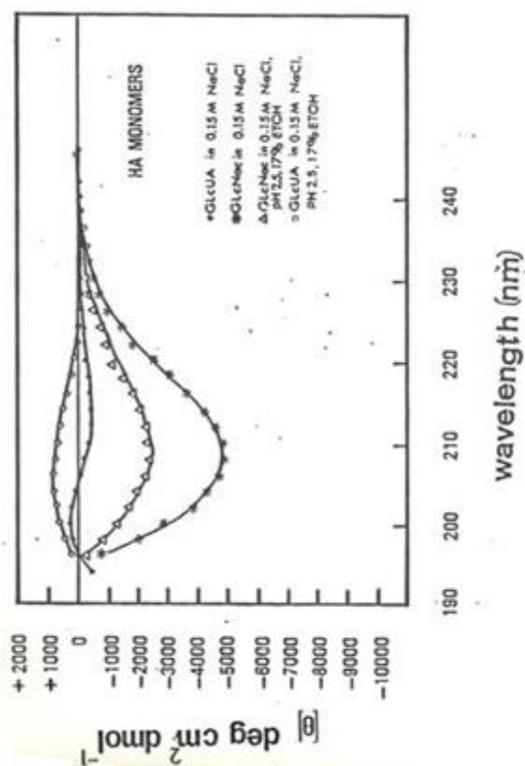


Figure 27. HA monomer spectra as a function of solvent type. Cell was 0.05 cm for all measurements.

HA oligosaccharide spectra as a function of solvent type

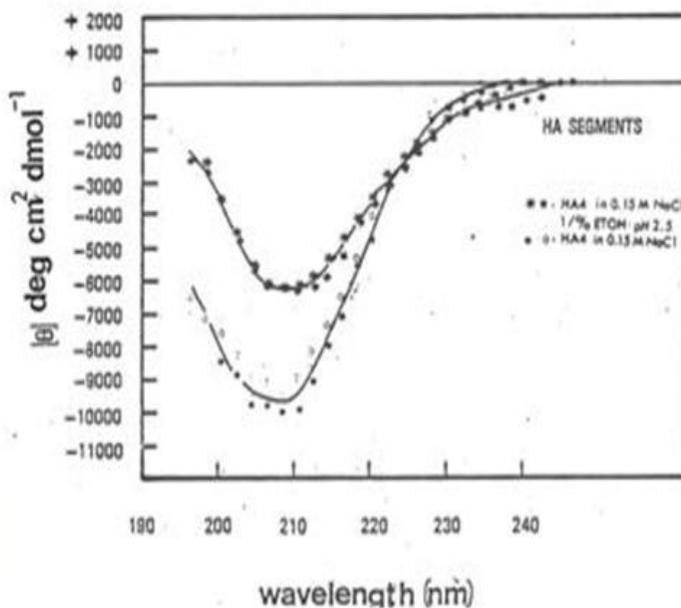


Figure 28. HA oligosaccharide spectra as a function of solvent type. Cell was 0.05 cm for all measurements.

CD Molar Ellipticity of Small HA
Segments and Monomers in 0.15M NaCl/
pH2.5/ETOH

NM.	GlcUA	GlcNAc	HA4	HA4(2)
194				
196	+270	-270		12240
198	+490	-860	-2280	-2610
200	+670	-1250	-3530	-3480
202	+790	-1700	-4460	-4710
204	+870	-1970	-5500	-5650
206	+860	-2240	-6120	-6080
208	+820	-2390	-6120	-6230
210	+760	-2360	-6020	-6300
212	+650	-2270	-5810	-6230
214	+580	-2000	-5290	-5860
216	+430	-1820	-4670	-5280
218	+290	-1520	-4050	-4270
220	+170	-1190	-3320	-3620
222	+50	-980	-2800	-3040
224	-40	-750	-2180	-2610
226	-120	-510	-1870	-2170
228	-170	-330	-1660	-1670
230	-190	-240	-1350	-1160
232	-210	-180	-1040	-1010
234	-220	-150	-830	-870
236	-210	-60	-420	-800
238	-180	-30	-210	-720
240	-140	-30	-100	-580
242	-100	0	0	-430
244	-80	0	0	-70
246	-70	0	0	0

Table 6. Monomer and oligosaccharide spectral data in SEA (salt/ethanol/acid) solvent. . Molar Ellipticity is listed as a function of wavelength. HA concentration was 187 and 268 (2) $\mu\text{g/ml}$ GlcUA in a 0.05 cm cell.

CD of HA Segments and Polymeric HA in 0.15M NaCl. Cell

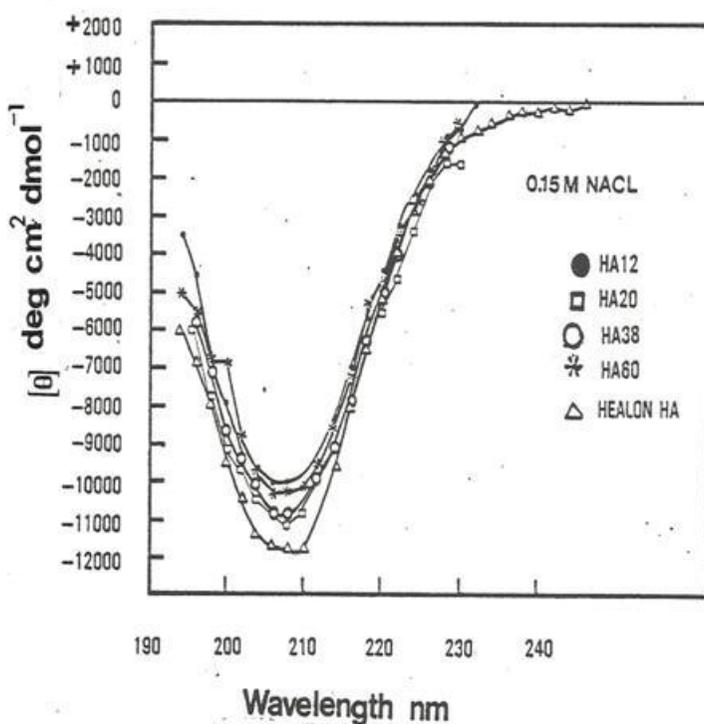


Figure 29. CD of HA segments and polymeric HA in 0.15M NaCl. Cell was 0.05 cm. Concentrations ranged from 21 to 250 $\mu\text{g/ml}$ GlcUA for segments.

CD of HA Segments and Polymeric HA in 0.15M NaCl, pH 2.5, and 17% ethanol (SEA)

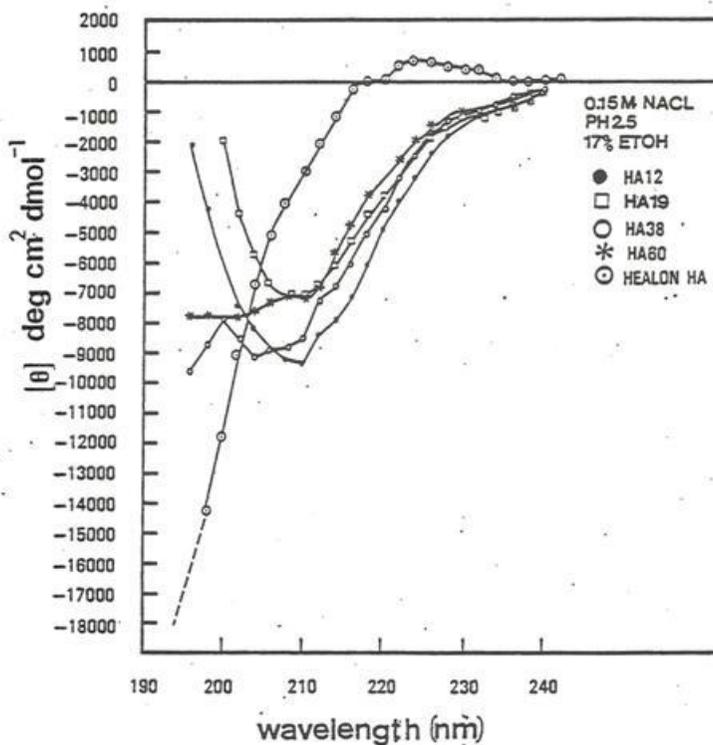


Figure 30. CD of HA segments and polymeric HA in 0.15M NaCl, pH 2.5, and 17% ethanol (SEA). Cell was 0.05 cm. Concentrations ranged from 22 to 160 $\mu\text{g/ml}$ GlcUA for segments.

CD Molar Ellipticity as a Function of Wavelength

NM.	HA12	HA15	HA20	HA38	HA60	Healon
194	-3570	-2430			-5000	
196	-4620	-2430	-5920	-5780	-5610	-6670
198	-7010	-3230	-7740	-7020	-6740	-7250
200	-7950	-3720	-9200	-8670	-6820	-8170
202	-9230	-4850	-9690	-9490	-8780	-9050
204	-9660	-6950	-10400	-10200	-9590	-9770
206	-10000	-7920	-10930	-10800	-10100	-10000
208	-10000	-8400	-11200	-10900	-10400	-10100
210	-9790	-8730	-10850	-10800	-10100	-9920
212	-9230	-8410	-9950	-9990	-9590	-9420
214	-8280	-7110	-9130	-9080	-8540	-8060
216	-6900	-6140	-7250	-7840	-7110	-6900
218	-5670	-4690	-6400	-6270	-5340	-5550
220	-4420	-4200	-5560	-5120	-4640	-4390
222	-3250	-3230	-4680	-4130	-3480	-3310
224	-2370	-2750	-3410	-2480	-2550	-2300
226	-1690	-1940	-2260	-1820	-1820	-1510
228	-1050	-1290	-1620	-1160	-890	-950
230	-600	-810	-1660	-740	-700	-650
232	0	-490	0	-500	-500	-420
234	0	-320	0	-410	-310	0
236	0	-160	0	0	0	0
238	0	0	0	0	0	0
240	0	0	0	0	0	0
242	0	0	0	0	0	0
244	0	0	0	0	0	0
246	0	0	0	0	0	0

Table 7. Average molar ellipticity of HA Segments and Healon HA in 0.15M NaCl. Cell path length is 0.05 cm.

Concentration Dependence of HA Segment (HA33) in 0.15M NaCl

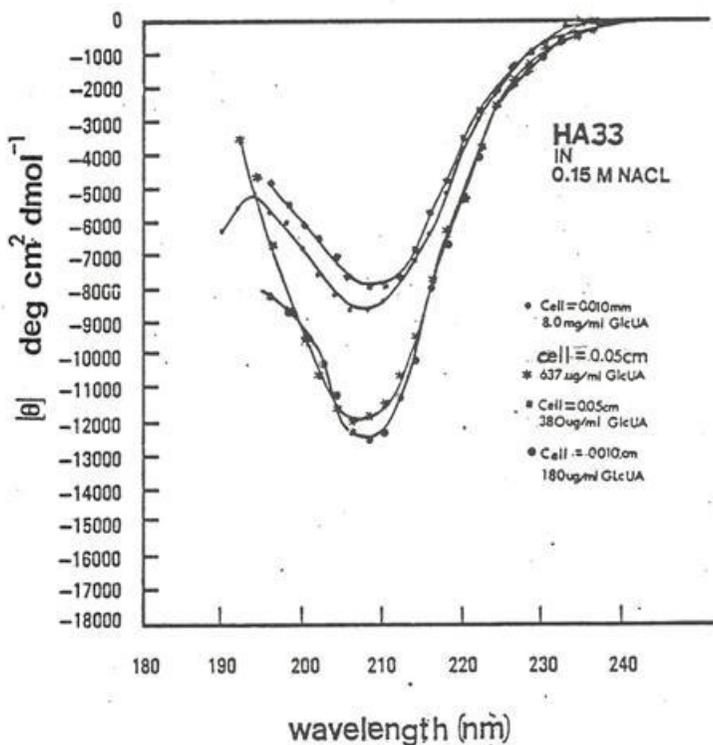


Figure 31. Concentration dependence of HA segment (HA33) in 0.15M NaCl. Samples were measured at the indicated concentrations and cell path lengths.

Concentration Dependence of HA Segment (HA33) in 0.15M NaCl, pH2.3, and 17% ethanol (SEA)

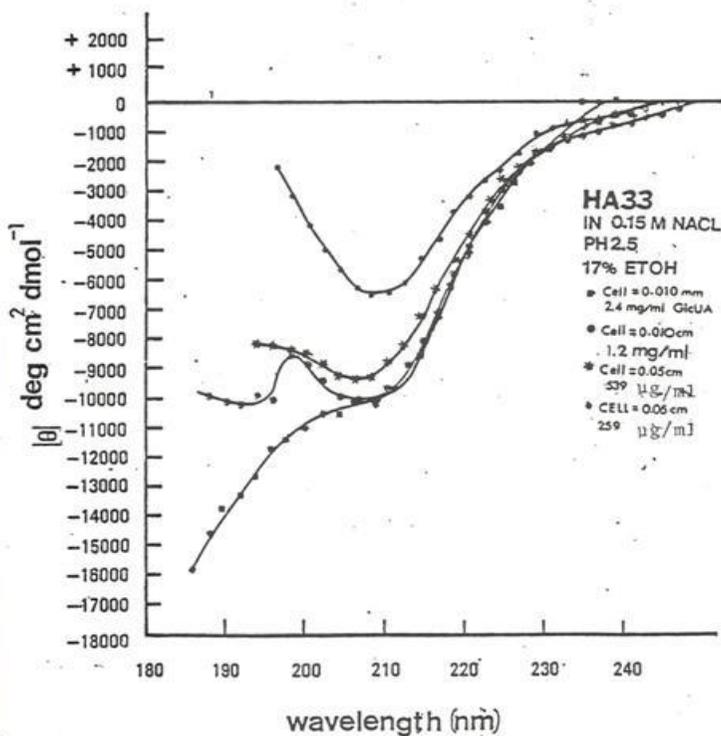


Figure 32. Concentration dependence of HA segment (HA33) in 0.15M NaCl, pH2.3, and 17% ethanol (SEA). Samples were measured at the indicated concentrations and cell path lengths.

CD Molar Ellipticity as a Function of Wavelength

NM.	HA12	HA15	HA20	HA38	HA60	Healon
194		0				-23100
196	-2170	0	-290	-9670	-7820	-20900
198	-4200	0	-1980	-8720	-7800	-14300
200	-6180	0	-4320	-7890	-7930	-11800
202	-7580	-7760	-5780	-8580	-7890	-9160
204	-8280	-10300	-6800	-9170	-7630	-6780
206	-8850	-11100	-7200	-8920	-7360	-5120
208	-9240	-10300	-7200	-8810	-7600	-4050
210	-9360	-9310	-6690	-8510	-7330	-2980
212	8470	-8020	-6180	-7310	-6870	-2020
214	-7900	-6730	-5340	-6860	-5770	-1190
216	-7260	-5690	-4490	-6050	-4780	-2380
218	-6120	-4660	-3870	-5060	-3850	0
220	-4910	-3620	-3250	-4200	-3600	+140
222	-4010	-2850	-2450	-3230	-2640	+600
224	-3250	-2590	-1930	-2430	-2000	+710
226	-2490	-1810	-1530	-1640	-1570	+600
228	-1850	-1290	-1280	-1280	-1250	+600
230	-1470	-780	-1130	-12101	-1010	+480
232	-1080	-3260	-990	-1030	-970	+360
234	-760	0	-880	-730	-760	+120
236	-510	0	-620	-550	-660	0
238	-450	0	-370	-480	-360	0
240	-260	0	-370	-300	-200	0
242	0	0	0	0	-160	0
244	0	0	0	0	-120	0
246	0	0		0	0	0

Table 8. Average molar ellipticity of HA Segments and Healon HA in 0.15M NaCl, pH2.5, and 17% ethanol. Cell path length is 0.05 cm.

CD Molar Ellipticity of HA33 as a Function of Molar Concentration in SEA

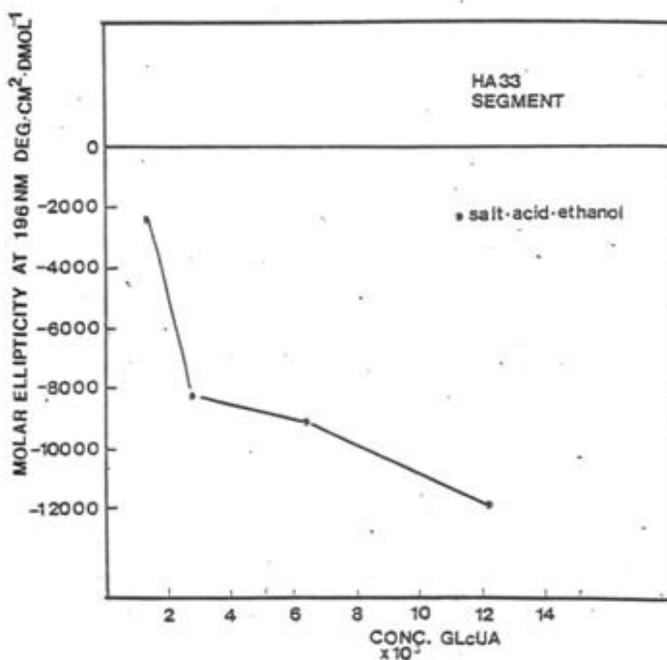


Figure 33. CD molar ellipticity of HA33 at 196 nm as a function of molar concentration in SEA.

Aggregation of Hyaluronic Acid Segments

[θ] 196 nm	Molar Conc. X 103	[MX]	Fract. of HA [M]	Log [MX]	Log [M]
11834	12.3	0.546	0.453	-2.17	-2.25
2397	1.3	0.022	0.604	-4.53	-2.42
9126	6.4	0.396	0.655	-2.60	-2.74
82218	2.8	0.345	0.978	-3.02	-2.88

Table 9. Data for HA33 calculated in 0.15M NaCl, pH 2.5, and 17% ethanol, according to a two state aggregation model. The molar ellipticity for the aggregated state is -20,000 deg cm² dmol⁻¹, while the molar ellipticity for the monomer is -2000 deg cm² dmol⁻¹.

Aggregation of HA33 in SEA

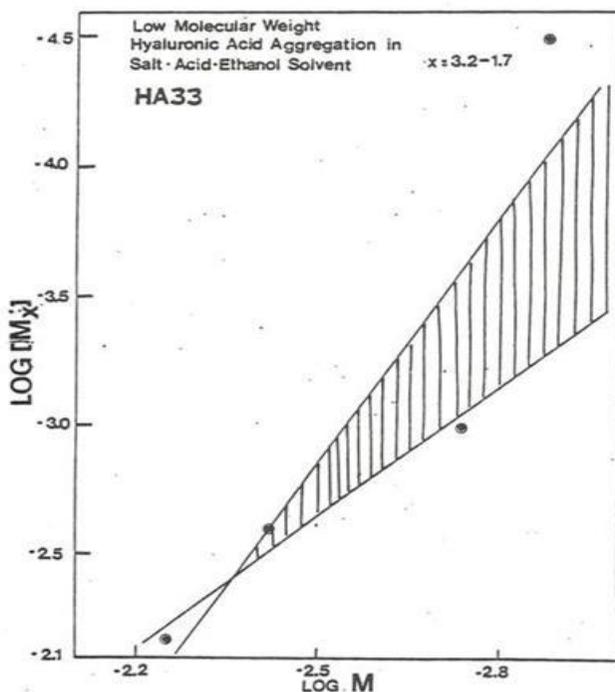


Figure 34. Aggregation of HA33 in SEA. The extent of aggregation (x) is determined by least squares fit. Using all the data points, $x=3.2$. Using data point for the three highest concentrations only, $x=1.7$. The uncertainty in the slope is shown by cross hatching the area between the two best-fit lines.

Effects of added segments on the spectral properties of polymeric HA in different solvents

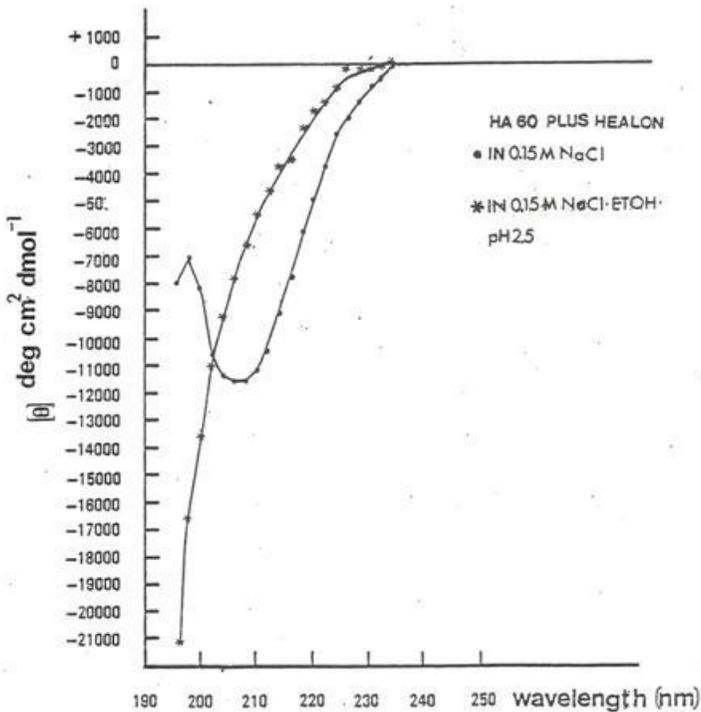


Figure 35. Effects of added segments on the spectral properties of polymeric HA in different solvents. All measurements were made in a cell of 0.05 cm path length.

Difference spectra for HA60 plus HA in 0.15M NaCl and in 0.15 M NaCl, 17% ethanol at pH 2.5

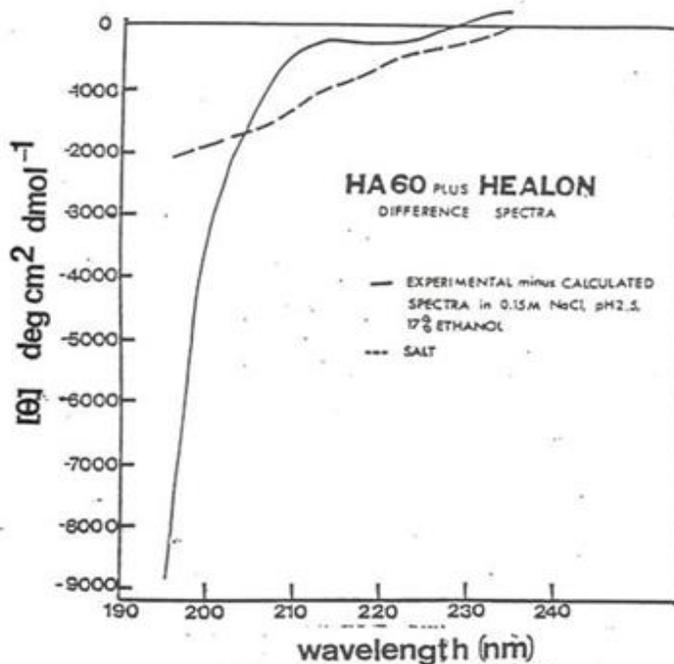


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Difference spectra for HA4 plus HA in 0.15M NaCl and in 0.15M NaCl, 17% ethanol at pH 2.5

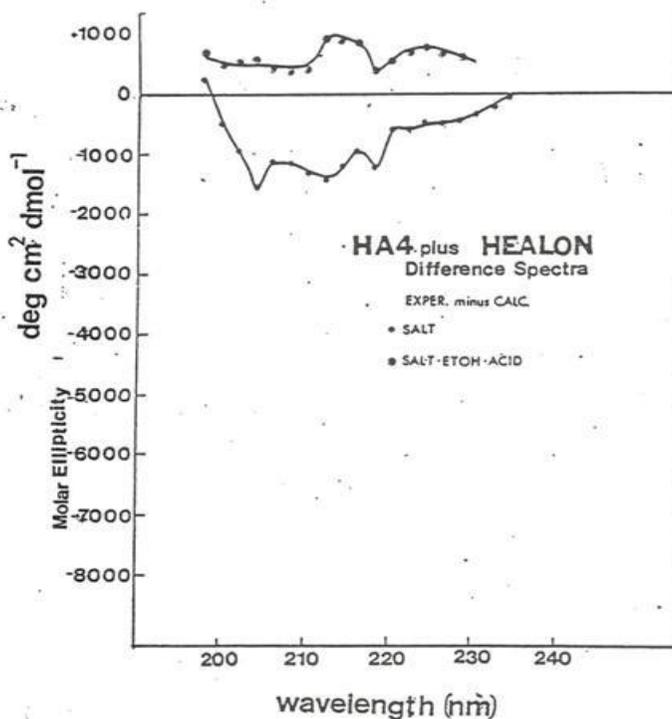


Figure 37. Difference spectra for HA4 plus HA in 0.15M NaCl and in 0.15M NaCl, 17% ethanol at pH 2.5. All measurements were made in a cell of 0.05 cm path length.

Chapter 5: General Conclusion Biological Role of Hyaluronic Acid Fragments

As previously mentioned in chapter 1, hyaluronic acid can be found in many different tissues. HA has been implicated as a major component of extracellular matrices through which cells migrate during embryonic tissue development and in regenerative processes. Changes in the level of hyaluronate accompany tumor growth¹¹⁵. Fluorescent probes have been

¹¹⁵ Toole, Bryan P., Chitra Biswas, and Jerome Gross. "Hyaluronate and invasiveness of the rabbit V2 carcinoma." *Proceedings of the National Academy of Sciences* 76, no. 12 (1979): 6299-6303.

used to probe and monitor changes in HA levels during tissue morphogenesis¹¹⁶ and binding sites for HA are present and have been identified on the surface of many cells¹¹⁷. These binding sites are responsible for a type of cell-cell adhesion, Addition of exogenous HA to cell suspensions can cause aggregation, which can be blocked by hyaluronidase. The enzyme may digest HA to fragments too small to be effective.

¹¹⁶ Knudson, Cheryl B., and Bryan P. Toole. "Fluorescent morphological probe for hyaluronate." *J Cell Biol* 100, no. 5 (1985): 1753-1758.

¹¹⁷ Underhill, C. B., Anne L. Thurn, and Brian E. Lacy. "Characterization and identification of the hyaluronate binding site from membranes of SV-3T3 cells." *Journal of Biological Chemistry* 260, no. 13 (1985): 8128-8133.

Maybe various degrees of digestion can permit various degrees or extent of cell-cell adhesion. The importance of HA fragments in biological systems has been documented¹¹⁸. In this particular study angiogenic, response (regeneration of new nerve tissue) was produced in chick chorioallantoic membrane as a function of chain length. Active fragments were between 4 and 25 disaccharides in length.

Silver and Swann³⁶ concluded from laser light scattering studies on vitreous and rooster comb HA that it is possible to reversibly alter the

¹¹⁸ West, DC, Hampson, IN, Arnold, I; and Kumar, S. "Angiogenesis induced by degradation products of hyaluronic acid." *Science* 228, (1985): 1324-1326.

apparent molecular weight of solutions of these polymers by modifying the nature of the interactions between chains. The authors further suggest that this phenomenon might be a mechanism by which tissue physical properties can be changed.

In general, not only the polymer, but also the breakdown products of hyaluronic acid might be biologically active. The high polymer might be more important in vitreous where it serves as a molecular sieve than on cell surfaces where it regulates cell-cell adhesion.

In view of these facts and the results of the present work, a model of HA in solution is proposed (Figure 38).

A Chain-Length Dependent Dynamic Model of Hyaluronic Acid in Solution

The model proposes that very short segments are incapable of intermolecular association with polymeric HA. Segments between 30 and 60 disaccharides can not only intermolecularly associate with high polymer but also can self-associate under certain forcing conditions. In this model, binding occurs through helical segments. Low molecular weight species are incapable of forming helical conformations of sufficient length, which are required for binding to local regions in the polymer known as microdomains. It has been previously reported by Sheehan et al⁴² that helical structures may be metastable and dynamic in nature based on

x-ray and infrared results on the stability of the double helix in the presence of various counterions. Local environmental factors may control the extent of interaction between polymer segments or between oligomers of sufficient length and polymer segments. The diffusion in tissue of certain ion types provides the driving force for inter-conversion of different helical structures⁴¹. Rapid transitions from liquid to solid character with increasing shear stress may also be related to double helical cross-links and stiff segments. ⁴⁴

The model is dynamic, and the network structure of hyaluronate can be altered as a function of interaction with added segment. However, the chain length requirement may vary

for a given micro-domain depending on local conditions. For example non-interacting species may become interacting species if their local concentration is increased or if driving forces are present. Figure 38 outlines the model outlined proposed in this work. Such a model is in agreement with the proposed biological roles of hyaluronic acid.

Oligomer Molecular Weight Dependent Modification of Segment Interaction in Polymeric HA

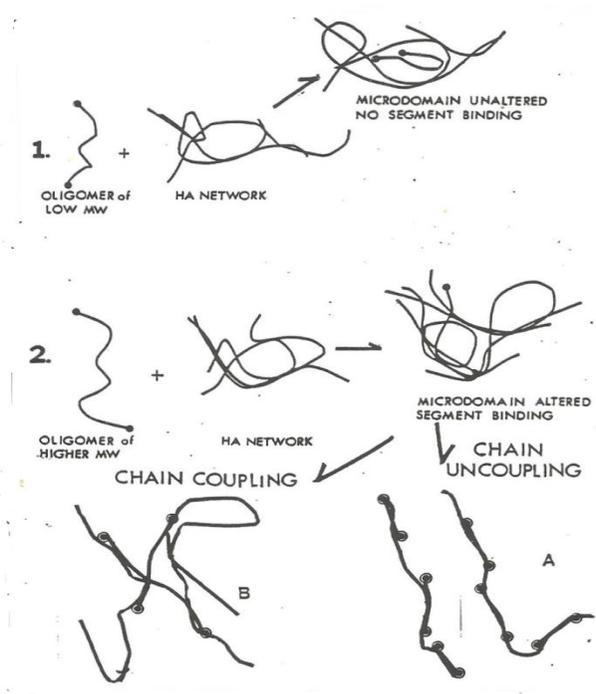


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