GLYCOSAMINOGLYCANS: STRUCTURE AND INTERACTION

Author: **Bireswar Chakrabarti**
Joon Woo Park*
Department of Retina Research
Eye Research Institute of Retina Foundation
Boston, Massachusetts

Referee: Eugene S. Stevens
Department of Chemistry
State University of New York
Binghamton, New York

INTRODUCTION

Glycosaminoglycans, commonly known as mucopolysaccharides, is a coined word from glycosamine (amino sugar) and glycans (polysaccharides). In a disaccharide unit, the amino sugar is always hexosamine, either D-glucosamine or D-galactosamine, mostly in the *N*-acetylated form; the other component is mainly D-glucuronic acid or L-iduronic acid, except that in keratan sulfate, D-galactose replaces an uronic acid. Glycosaminoglycans, except hyaluronic acid, contain sulfate groups, which are linked by an ester bond to hydroxyl groups. In addition, in some cases (heparin and heparan sulfate), acetamido groups are partly *N*-sulfated. Because of their carboxyl and sulfate groups, these biopolymers are highly negatively charged at physiological condition. The term *acidic mucopolysaccharides,* or *mucopolysaccharides,* was introduced by Meyer in the 1930s to describe hexosamine-containing polysaccharides of animal origin. Glycosaminoglycans, with the possible exception of hyaluronic acid, are present in tissues as segments of larger macromolecules, the proteoglycans, which consist of carbohydrate chains (the glycosaminoglycans) covalently linked to protein.

Interest in the structure-function relationship of biomacromolecules has promoted extensive physicochemical studies of the glycosaminoglycans. Hydrodynamic properties, X-ray diffraction, spectroscopy, and interaction behavior have been the main avenues of investigation, but the relation of the data to biological function is still largely conjectural. The main thrust of this presentation will be a review of the more recent studies on structure and interaction properties of glycosaminoglycans, and the proposed correlations with biological function.

Earlier studies on the distribution and structure of glycosaminoglycans are well described by Mathews.[1] Physicochemical properties with details of polyelectrolyte behavior can be found in an excellent article by Bettelheim.[2] Reviews by Stone[3,4] of the conformational aspects of hexose polysaccharides in solution included the earlier chiroptical studies of glycosaminoglycans. A detailed review of the physiological function of glycosaminoglycans has been recently published.[5] Earlier results on structure and various properties of glycosaminoglycans were described by Muir,[6] Rogers,[7] Schubert,[8] Laurent,[9] and Sharon.[10] One glycosaminoglycan, heparin, has been studied extensively; its chemistry and pharmacology have been reviewed by Ehrlich and Stivala[11] and Engelberg.[12] The techniques of isolation and characterization of glycosaminoglycans were detailed by Rodén et al.[13]

* Present address: Senior Research, Korean Research Institute of Chemical Technology, Dae-Duk, Choong-Nam, Korea.

OCCURRENCE, PRIMARY STRUCTURE, AND FUNCTION

Glycosaminoglycans occur in many vertebrate connective tissues and fluids, such as skin, bone, cartilage, arterial walls, umbilical cord, vitreous humor, cornea, and synovial fluids.[1] They are also found in cell membranes and in the cerebral cortex of the brain. In some cases, glycosaminoglycans occur within cells, primarily in mast cells (heparin) and in circulating cells such as granulocytes and platelets; they have also been detected in cell nuclei.[14] Glycosaminoglycans have also been reported in invertebrates.[15] It is generally accepted that the relative amount, molecular size, and type of glycosaminoglycans are tissue specific, as demonstrated by Toledo and Dietrich[16] who reported the composition of sulfated glycosaminoglycans of various mammalian tissues. The pattern of distribution of glycosaminoglycans in a specific tissue or fluid changes with maturation and aging. Especially large changes were observed during growth and in cancer tissues.[17] It has been suggested that disturbances in the normal distribution of these polysaccharides lead to serious clinical abnormalities that are characteristic of various diseases such as rheumatoid arthritis and skin and ocular diseases. Abnormalities in the metabolism of mucopolysaccharides in man have been recognized for some time, and it was found quite early that these diseases, known collectively as *mucopolysaccharidoses,* are of genetic origin. A detailed discussion of these conditions are beyond the scope of this review; interested readers are referred to Sharon.[10]

The common monosaccharide units of glycosaminoglycans are shown in Figure 1.

D-galactose in keratan sulfate has the same configuration as D-galactosamine except that it bears a hydroxyl group instead of an amine group. The numbering of the parent sugar ring system and the glycosidic linkage notations are demonstrated in Figure 2.

The classification of glycosaminoglycans is mainly based on the early work of Meyer's group. The composition and linkage of the repeating disaccharide units and the major sources of glycosaminoglycans are shown in Table 1.

The general name of each glycosaminoglycan can be derived from its chemical composition and the linkages of its disaccharide units. For example, the repeating unit of hyaluronic acid (Figure 3) can be called (1 → 4)-O-β-D-glucopyranosyluronic acid-(1 → 3)-2-acetoamido-2-deoxy-β-D-glucopyranose.

The main biological role of glycosaminoglycans is thought to be structural, rather than chemical, providing toughness and flexibility in the connective tissues of animals. Because of the highly charged nature of the polymers, it is believed that the molecules may also play important roles in the control of electrolytes and water in extracellular fluids. The polyanionic nature of glycosaminoglycans may also provide electrostatic interaction with polycationic molecules or the positively charged region of a macromolecule. Due to their high molecular weight and the flexibility of the linear molecular chains in solution, glycosaminoglycans occupy a much larger volume or domain than would a molecule of comparable size in a solid sphere of the same molecular volume. This effect may appear as large excluded volume of the polymers in solution and as a consequence, a diffusional barrier to other macromolecules. In the following sections, we will describe the primary structure and specific functions of each glycosaminoglycan in detail.

Hyaluronic Acid

Since the first isolation of hyaluronic acid from the vitreous humor of cattle eyes by Meyer and Palmer,[18] the polymer has been found to be widely distributed in various connective tissues, synovial fluids, skin, and vitreous, and in some bacteria.[1,19,20] In some tissues, hyaluronic acid is the major glycosaminoglycan present. Examples are the vitreous of the eye and the synovial fluid in joints, bursae, and tendon sheaths. In

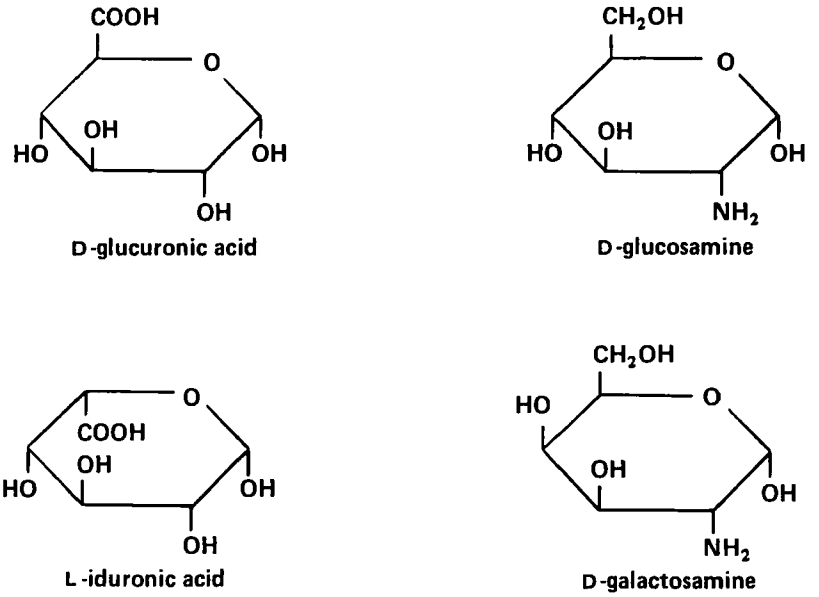


FIGURE 1. Monosaccharide units of glycosaminoglycans. Amine groups of hexosamine moieties are either *N*-sulfated or *N*-acetylated.

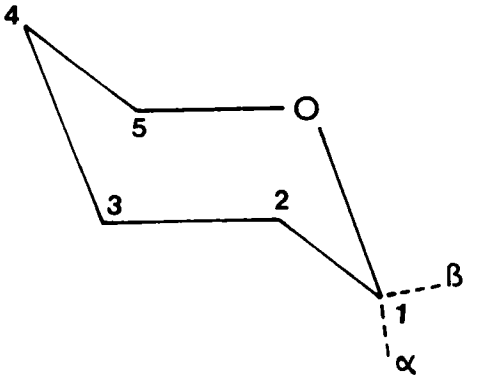


FIGURE 2. Common numbering of parent hexose ring. Methylene or carboxyl carbon linked to C_5 is numbered 6.

TABLE 1

Structure of Glycosaminoglycans[a]

Compound	Molecular weight	Components[b]	Location of sulfate groups	Linkage	Major source
Hyaluronic acid	1—5 × 10^6	*N*-Ac-Glu	—	β (1→4)	Vitreous humor, synovial fluid, umbilical cord, rooster comb
		Glu	—	β (1→3)	
Chondroitin	2—5 × 10^4	*N*-Ac-Gal	—	β (1→4)	Cornea
		Glu	—	β (1→3)	
Chondroitin 4-sulfate	2—5 × 10^4	*N*-Ac-Gal	4	β (1→4)	Cartilage, bone, skin, aorta
		Glu	—	β (1→3)	
Chondroitin 6-sulfate	2—5 × 10^4	*N*-Ac-Gal	6	β (1→4)	Heart valves, cartilage
		Glu	—	β (1→3)	
Dermatan sulfate	2—5 × 10^4	*N*-Ac-Gal	4	β (1→4)	Skin, blood vessels, heart valves
		Idu	—	α (1→3)	
Heparin	1—2 × 10^4	Glucosamine[c]	3,6,*N*	β (1→4)	Lung, mast cells, liver, skin
		Idu (major)	2	α (1→4)	
		Glu (minor)	—	β (1→4)	
Heparan sulfate	0.2—1 × 10^4	Glucosamine[c]	3,6,*N*	β (1→4)	Cell surfaces, liver, lung, blood vessels, kidney
		Idu (minor)	2	α (1→4)	
		Glu (major)	—	β (1→4)	
Keratan sulfate	0.5—2 × 10^4	*N*-Ac-Glu	6	β (1→3)	Cornea, cartilage
		D-galactose	6	β (1→4)	

[a] The information in this table is idealized to some degree. Wider variations in molecular weight, components, and degree of sulfation have been reported.

[b] *N*-Ac-Glu, *N*-acetyl-D-glucosamine; *N*-Ac-Gal, *N*-acetyl-D-galactosamine; Glu, D-glucuronic acid; Idu, L-iduronic acid.

[c] Amine group of D-glucosamine is either *N*-sulfated or *N*-acetylated.

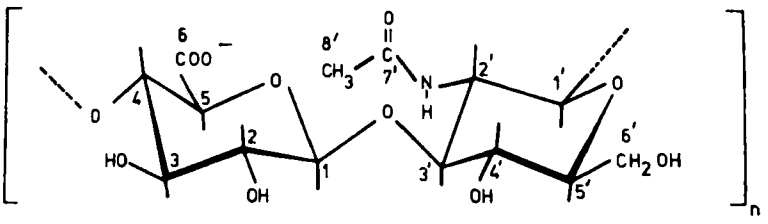


FIGURE 3. Disaccharide repeating unit of hyaluronic acid showing β (1→ 4) and β (1 → 3) linkages. Numbers 1-6 designate the β-D-glucuronic acid carbon atoms, and numbers 1′-8′, the *N*-acetyl-β-D-glucosamine carbon atoms. Glycosidic linkages 1 → 4 and 1 → 3 are diequatorial. Pyranose rings are in chair conformation. Hydroxyl, carboxyl, hydroxymethylene, and *N*-acetylamino groups are equatorial.

other intercellular matrices, hyaluronic acid shares the interfibrillar (collagen fibrils) space with varying amounts of other glycosaminoglycans and their proteoglycans. These matrices comprise the subcutaneous, perimuscular, perineural, and perivascular connective tissues, and the surface layer of articular cartilage. The molecular weight of hyaluronic acid, 10^5 to 10^7 depending on the source, is the highest among the glycosaminoglycans. Physical characteristics of hyaluronic acid from different tissues are well documented by Rogers[7] and Laurent.[9] The polymer is noted for its large hydrodynamic volume and elastoviscous properties. Because of these physical characteristics, a concept[21,22] has been formulated regarding the possible role of hyaluronic acid in biological systems. However, it must be emphasized that most of the roles assigned to hyaluronic acid are highly speculative and that these hypotheses are supported by very little experimental evidence. It has been postulated that the molecule acts as an absorbent to mechanical stress, thereby protecting the cells from mechanical shock and vibration. It was also proposed that at the interface between solid and liquid matrices, such as the surfaces of tendons, tendon sheaths, and synovial membranes, hyaluronic acid serves as a lubricating agent. Recently, hyaluronic acid has been considered[23,24] to function as a mechanoelectrical transducer. The presence of hyaluronate in the culture medium has been shown to decrease lymphocyte stimulation.[25] It was also suggested that the molecule plays a regulatory role in the synthesis of proteoglycans in chondrocytes.[26-28] However, Yamagata et al.[29] recently reported that the chondrocytes cultured in the suspension system did not appear to be affected by hyaluronic acid.

The primary structure (Figure 3) of hyaluronic acid was established by the work of Meyer's and Jeanloz's groups. The polymer is noted for its homogeneity in primary structure. Although common sources for isolation of hyaluronic acid are human umbilical cord and rooster comb, no variation in primary structure — except for mol wt — has been reported when the molecule is prepared from other tissues.

Chondroitin and Chondroitin Sulfates

The primary structure of chondroitin differs from that of hyaluronic acid only in that its hexosamine moiety is galactosamine instead of glucosamine. It was isolated by Davidson and Meyer[30] from bovine cornea and was thought to be the precursor of chondroitin sulfates. Chondroitin is also easily prepared by desulfation of chondroitin sulfates.[31]

Chondroitin 4-sulfate (also referred to as chondroitin sulfate A) and chondroitin 6-sulfate (chondroitin sulfate C) differ only in the position of the ester sulfate group on the galactosamine moiety of chondroitin. Chondroitin 4-sulfate, which bears a sulfate group in the C-4 position, was first isolated from cartilage more than a century ago. Isolation of chondroitin 6-sulfate from umbilical cord was reported by Meyer and Palmer;[32] the position of its sulfate group was determined by several groups in the 1950s. It was once generally believed that the glycosaminoglycans in the vitreous body were exclusively hyaluronic acid. However, Breen et al.[33] showed the presence of galactosamine-containing proteoglycan in human vitreous. Chondroitin 4-sulfate and undersulfated heparan sulfate, but not chondroitin 6-sulfate, were isolated from the vitreous body.[34] Chondroitin sulfates are the glycosaminoglycan most widely distributed among animal tissues and fluids.

The descriptions of chondroitin 4-sulfate and 6-sulfate as homogeneous polymers of glucuronic acid and *N*-acetylgalactosamine 4-sulfate and 6-sulfate, respectively, are only idealized structures; considerable departures from these representations have been shown. Bettelheim and Philpott[35] isolated partially sulfated chondroitin 4-sulfate from bovine trachea; the structure was proposed to be stereospecifically sulfated on every third galactosamine moiety.[36] Juvani et al.[37] and Liau et al.[38] isolated undersulfated chondroitin 4-sulfate from human plasma and from rat rib, respectively. Suzuki[39]

showed that chondroitin sulfate from shark cartilage is oversulfated; most of the ester sulfate is at the C-6 position of galactosamine, but part of the sulfate residue is at the C-2 and C-3 positions of the glucuronic acid moiety. This moleucle was named chondroitin sulfate D. A similar type of oversulfated chondroitin sulfate has been reported by several groups to occur in elasmobranch cartilage.[1] Another type of oversulfated chondroitin sulfate, which differs from chondroitin sulfate D in optical rotation and infrared spectrum, was reported by Mathews et al.[40] from cartilage of squid and of horseshoe crab. Studies by Kawai et al.[14] and Suzuki et al.[42] showed that this type of chondroitin sulfate contains 4,6-disulfated galactosamine residue; it was termed chondroitin sulfate E by the latter group. Habuchi et al.[43] showed that a D-glucose unit is substituted in the C-6 position of one or both galactosamines of a tetrasaccharide unit of chondroitin sulfate E. Another disulfated disaccharide unit, O-sulfated in either C-2 or C-3 of the glucuronic acid moiety and in C-4 of the galactosamine component, was isolated from king crab cartilage, and named *chondroitin sulfate K*.[44] The occurrence of a copolymer of chondroitin 4-sulfate and chondroitin 6-sulfate has also been reported.[38,45]

The ratio of the various chondroitin sulfates differs in different cartilaginous tissues of the same individual.[46] Generally, large amounts of chondroitin sulfates were found in neonate and in tumoral tissues, both of which are in exponential phases of growth.[17,47,48] Dietrich and co-workers[48] suggested that chondroitin 4-sulfate and 6-sulfate are probably involved in cell differentiation and determine some of the specific cell properties such as recognition and/or adhesiveness. The ion binding, especially of calcium, properties of chondroitin sulfates drew extensive attention in connection with mineralization of connective tissues; this will be discussed in detail in a later section.

Dermatan Sulfate (Chondroitin Sulfate B)

Dermatan sulfate, first isolated from pig skin by Meyer and Chaffee[49] in 1941, differs from chondroitin 4-sulfate in that the uronic acid moiety is L-iduronic acid, rather than D-glucuronic acid. This compound was called chondroitin sulfate B or β-heparin in the earlier literature.

Like chondroitin sulfates, dermatan sulfate shows structural heterogeneity. Fransson's group studied this matter extensively and showed that D-glucuronic acid (instead of L-iduronic acid) and *O*-sulfated L-iduronic acid are integral parts of dermatan sulfate.[50-53] Similar results were reported by Habuchi et al.[54] and Michelacci and Dietrich.[55] The repeating period of dermatan sulfate containing C-2 or C-3 sulfated iduronic acid from pig skin was termed dermatan sulfate D,[51] and the term chondroitin sulfate H was given to units containing C-4 and C-6 disulfated galactosamine moiety isolated from hagfish notochord.[42,56]

Heparin and Heparan Sulfate

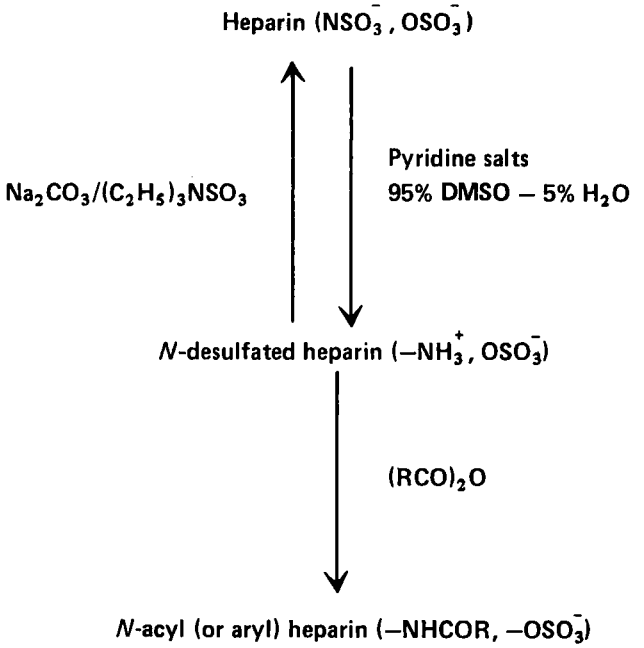
Heparin is a highly sulfated glycosaminoglycan composed of partially sulfated uronic acid (either α-D-glucuronic or β-L-iduronic acid) and α-D-glucosamine joined by 1 → 4 bonds. It shows very high anticoagulant activity; it is found in liver, muscles, lung, heart, kidney, spleen, skin, and blood. It is also an antilipemic agent. The structure of the molecule and its relation to the biological actions have been extensively studied (for details on chemistry and biological activities, see Reference 11). The primary structure of heparin is still not fully known. However, it is generally believed that the molecule does not have a homogeneous chemical structure with respect to uronic acid composition and degree of *N*-sulfation and *O*-sulfation. (Chondroitin sulfates and dermatan sulfate also show some heterogeneity of structure, but much less than that of heparin).

The most distinct difference of heparin (and heparan sulfate) from other glycosa-

minoglycans is the presence of an *N*-sulfated group (sulfoamido) in the hexosamine moiety. The glucosamine component of heparin is nearly fully *O*-sulfated at the C-2 position. The *O*-sulfate group may also be located at the C-2 position of the uronic acid and the C-3 position of the hexosamine moiety. Various degrees of *N*-acetylation, depending on the source and reaching as high as 30% from whole tissues, have been found.[57,58]

No clear-cut structural difference seems to exist between heparin and heparan sulfate, which was previously named heparin monosulfate or heparitin sulfate. Generally, heparan sulfate is obtained from by-products of heparin preparation, and shows higher *N*-acetylation, higher glucuronic acid content, and less *O*-sulfation than heparin, and virtually no anticoagulant activity.[59-62] It has been suggested that heparan sulfate may be a block copolymer with segments of multiple sequences of *N*-acetylglucosamine.[63,64] Cifonnelli and King[61] showed that ester sulfate occurs predominantly on the iduronic acid moieties rather than on the *N*-sulfated hexosamine units, and that the sulfated iduronic acid residues are almost completely in single sequences.

Heparin was chemically modified for studies of its biological function and molecular conformation.[65-68] The following illustrates the commonly used techniques.



Hydrolysis of heparin under mild acidic condition was also widely used for *N*-desulfation, and it has been suggested that the hydrolysis of *N*-sulfate is accompanied by some cleavage of glycosidic linkages and *O*-sulfate groups. When chondroitin sulfate is hydrolyzed under 0.04*M* HCl at 100°C for 1.5 hr, the polymer is degraded and 45% of the *O*-sulfate groups undergo cleavage.[69] Under similar conditions, no degradation of heparin was detected, but *N*-desulfation did occur. Carboxyl groups of both the iduronic and glucuronic acid moieties of heparin, as well as of the other glycosaminoglycans, are reduced by treatment with sodium borohydride.[63,70]

Removal of the *N*-sulfate group from heparin resulted in loss of its anticoagulant activity.[65,71-73] However, high *N*-sulfate content of native heparin of different origins does not necessarily correlate with high anticoagulant activity. Danishefsky et al.[74] studied the effects of various modifications of heparin on its antithrombin III-enhanc-

ing activity. The free carboxyl groups of heparin were found to be important, although not crucial, for its binding to antithrombin III. In contrast, the *N*-sulfate group is crucial for this binding. Treatment of heparin with 4% acetic acid at 37°C for a day resulted in loss of almost all of the anticoagulant properties, but no substantial decrease in hypolytic activities.[71]

Heparan sulfate has been identified as the major glycosaminoglycan isolated from kidney, liver, and ileum tissues.[16] The polymer was also isolated from cell surface or plasma membrane fractions of various cells,[75-77] and from ascites hepatoma cells and fluids.[78]

Keratan Sulfate

Keratan sulfate, also called keratosulfate, is a unique glycosaminoglycan in that it has galactose rather than uronic acid in its primary structure. It constitutes about half of the glycosaminoglycan content of bovine cornea.[79] The keratan sulfate from cornea is usually referred to as keratan sulfate I to differentiate it from skeletal keratan sulfate II, which differs slightly in its minor constituents, sialic acid and pentose, in the linkage region. Keratan sulfate is present mainly in cartilage as an integral part of the proteoglycan monomer molecules; both chondroitin sulfate (major) and keratan sulfate (minor) side chains are believed to be covalently attached to a central protein core.[1,80] Vitello and co-workers[81] isolated a keratan sulfate-like glycosaminoglycan from the cerebral cortex of rat brain, and showed that it tripled in amount during the rapid growth phase from 1 to 3 months of age and subsequently decreased to a negligible quantity in the senescent rat, aged 25 months.

The C-6 position of the glucosamine component of keratan sulfate is *O*-sulfated. However, the sulfate content of keratan sulfate varies widely, depending on the source or even within a single tissue.[82]

HYDRODYNAMIC PROPERTIES

Consideration of glycosaminoglycan structure can be conveniently divided into two parts. In this section, we will review the studies of the hydrodynamic properties of these polymers, which have furnished information regarding gross structural features such as molecular size and shape. A knowledge of the gross structure permits the distinction between compact and extended structure and also between rodlike (e.g., helical) and random-coil-like configurations of a macromolecule. The information on size and shape is also helpful in formulating models for the conformational changes that accompany denaturation or other interactions. In later sections, we will consider various aspects of internal configuration of glycosaminoglycan molecules and their three-dimensional structure.

The interpretation of hydrodynamic data in terms of size and shape requires a knowledge of the molecular weight, M, of the polymer. Hence the principal objective of early investigations was to establish methods of mol wt determination. A variety of methods — hydrodynamic (osmotic pressure, viscosity, sedimentation-diffusion, flow birefringence, etc.), light scattering, and thermodynamic — provide, in principle and even in practice, a value of M. If the system is heterogeneous, the various methods give different types of molecular weight averages[2] — osmotic pressure measurements, for example, give a number average and light scattering data give a weight average. Heterogeneity of molecular weights has been found among all glycosaminoglycans.

Different approaches[83] have been taken in the development of theories of the hydrodynamic behavior of particles of various shapes, such as rigid (α-helical polypeptide) or flexible (polystyrene) chain molecules. Glycosaminoglycans have been

considered[1,2,5,9,11] as more or less flexible molecules. Within the framework of theories concerning flexible chain molecules, the properties of dilute polymer solutions, such as average molecular dimensions, second virial coefficients, and intrinsic viscosities, may be expressed in terms of two basic parameters:[84] mean-square end-to-end distance, $\langle R^2 \rangle_o$, and excluded-volume parameter, z. The parameter z is proportional both to the effective excluded volume for a pair of chain elements at infinite dilution and also to the square root of the number of elements in the chain. The theta state is defined as a sort of ideal state, in the sense that at that state the volume effect apparently vanishes. The major problem in the theory is to derive the interrelationship between the dilute solution properties and the parameters, $\langle R^2 \rangle_o$ and/or z, in particular for linear flexible chains. The problem, however, is ultimately concerned with exploration of the dependence of dilute solution properties on molecular weight. Although there is a large accumulation of hydrodynamic data on glycosaminoglycans, few investigators have analyzed the values in terms of these theories of polymer solutions as has been done for proteins, polypeptides, and synthetic polymers. Hence, no attempt is made in this review to describe all the theories even in a cursory manner. The equations that are commonly used for analysis of hydrodynamic measurements will be cited here without derivations. It is hoped that this is adequate for our purpose. We also exclude such values as radius of gyration and end-to-end distance of glycosaminoglycans; for these quantities readers are referred to earlier reviews.[2,3] The overall stiffness or flexibility of glycosaminoglycans has been assessed from the relation of intrinsic viscosity to mol wt.[1,2,5,9,11] Among the flexible polymers, the intrinsic viscosity depends, as expected, on structure; the more extended the polymer chain, the higher is the intrinsic viscosity. The intrinsic viscosity is also strikingly dependent on the medium in which the polymer is dissolved. These observations suggest the complexities of the hydrodynamic properties of macromolecules and emphasize the need for coordinating these measurements with other physicochemical methods. In the last few years, studies have been made in this direction. The present review will thus include mostly the results of recent investigations that have successfully correlated the hydrodynamic and optical properties of glycosaminoglycans in solution.

The hydrodynamic properties of a dissolved macromolecule may be considered as resulting from rotational and translational frictions. Three experimental techniques — viscometry, sedimentation-diffusion, and birefringence — are commonly used to characterize the hydrodynamic properties and hence the molecular parameters of the macromolecules.

Viscosity of a solution is usually analyzed by Huggins equation:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \qquad (1)$$

where the specific viscosity is defined as $\eta_{sp} = (\eta_{solution}/\eta_{solvent})-1$. The plot of reduced viscosity $[\eta_{sp}/c]$ against concentration c yields a straight line, when concentration is low, and intrinsic viscosity $[\eta]$ and interaction parameter k′ are calculated from the plot. A semiempirical relationship of Mark-Houwink (Equation 2) relates the intrinsic viscosity and molecular weight of the solute molecule.

$$[\eta] = kM^{\alpha} \qquad (2)$$

The parameter α is the shape factor of the molecule; it is 0.5 for a compact sphere, 0.75 for a random coil, and more than 1 for an extended rigid rod.

The polyelectrolytic nature of glycosaminoglycans results in a complex behavior of viscosities of the solutions as a function of polymer concentration and of ionic strength

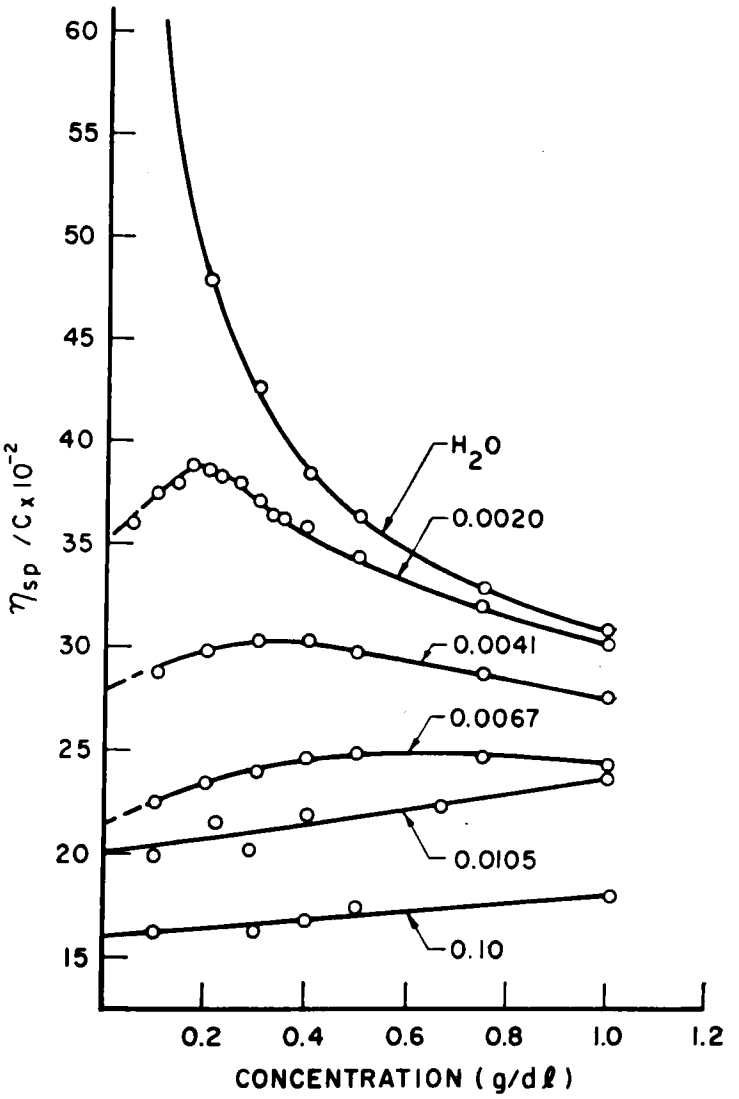


FIGURE 4. Reduced viscosity vs. concentration of heparin, exhibiting typical polyelectrolyte behavior in water and NaCl solutions of various molarity, measured at 25°C. Other glycosaminoglycans show similar behavior. (From Liberti, P. A. and Stivala, S. S., *Arch. Biochem. Biophys.*, 119, 510, 1967. With permission.)

of the environment, rather than the simple behavior expected from Equation 1. Figure 4 illustrates the viscosity behaviors of heparin at various added NaCl concentrations.

As evident from the figure, Equation 1 is inapplicable for solutions of low ionic strength to obtain [η] from extrapolation to zero concentration. This difficulty may be removed by using the technique of isotonic dilution, in which the total ionic contribution of the polyelectrolyte and the supporting electrolyte is kept constant or by experimenting at a higher concentration of supporting electrolyte.

Fuoss and Strauss[85] quantitatively described the viscosity behavior of a polyelectrolyte in an aqueous solution, where it fails to obey the Huggins equation:

$$\frac{\eta_{sp}}{c} = A/(1 + B\sqrt{c}) \qquad (3)$$

where A and B are constants characterizing the system and can be determined from the plot of c/η_{sp} versus $\sqrt{c}$. A is shielded intrinsic viscosity (commonly shown as $[\eta]\infty$) of the solute polyelectrolyte in the solvent environment. Yuan and co-workers[86] expressed the interaction parameter as $B = 5.22\sqrt{P}/a^2$ (P is molecular weight/length, and a is the equivalent freely jointed segment length of the molecule when the backbone of the polyion is represented by a Gaussian coil of N segments). However, it was observed that for an exceedingly dilute solution, the reduced viscosity (η_{sp}/c) of a polyelectrolyte decreases with further dilution, resulting sometimes in a zero or negative value of $[\eta]\infty$.[87] Liberti and Stivala[87] modified Equation 3 to include a third parameter k″:

$$\frac{\eta_{sp}}{c} = A/[1 + B(\sqrt{c} - k''c)] \quad (4)$$

The parameter k″ is obtained from adjustment of the trial k″ value until the plot gives the linear curve expected from the equation. (The equations describing viscosity behavior (Equations 2,3, and 4) are limiting equations, and extreme care must be used in extrapolating limited experimental data measured at rather high concentration).

Sedimentation velocity (s) of a macromolecule is generally measured in an ultracentrifuge equipped with a proper optical system. Like viscosity, sedimentation velocity depends on concentration through solute-solute interaction, and the sedimentation coefficient of an infinitely dilute solution s° is determined to characterize the macromolecules.

$$1/s = 1/s^\circ (1 + k_s c) \quad (5)$$

The sedimentation coefficient (s°) is related to the frictional coefficient (f°), partial specific volume ($\bar{v}$) of the solute molecule, density of solvent (ρ), and mol wt (M) of the solute molecule.

$$s^\circ = M(1 - \bar{v}\rho)/Nf^\circ \quad (6)$$

where N is Avogadro's number.

Diffusion coefficients are usually obtained by measuring the concentration gradient (from refractive index gradient measurement) at different times during ultracentrifugation. Extrapolation of the diffusion constant to infinite dilute solute concentration gives the diffusion coefficient (d_o) of the solute in the solute environment, which is inversely proportional to f°.

$$d_o = RT/Nf^\circ \quad (7)$$

The mol wt of the solute macromolecule can be obtained from s_o and d_o, using independently measured $\bar{v}$ and ρ values. The reported values of sedimentation-diffusion, viscosity, and other molecular parameters of glycosaminoglycans are tabulated in Table 2.

Although most investigators used the Huggins equation (Equation 1) to study viscosity of glycosaminoglycans, Stivala's group used Equation 3 or 4 for heparin to obtain viscosity parameters in low or zero salt solutions. Liberti and Stivala[88] obtained $[\eta]_\infty$ of various molecular weight fractions of heparin using Equation 4; k″ was 0.25 for all fractions. They showed the Mark-Houwink parameter as a function of salt concentration; in water, and in 0.01 M, 0.1 M, and 0.5 M NaCl, the values were 1.59,

TABLE 2

Hydrodynamic Parameters of Glycosaminoglycans

				Sedimentation-diffusion				Viscosity			
Material	Source	Solvent[a]	M.W. × 10^{-4}	$s^{\circ}_{20,w} \times 10^{13}$ (sec)	K_s	$d^{\circ}_{20,w} \times 10^{7}$ (cm^2/sec)	$\bar{V}$ (ml/g)	$[\eta]$ (dl/g)	$K_m \times 10^{-4}$	a	Ref.
Hyaluronic acid	Vitreous	0.2 *M* NaCl	7.7—170	2.46—7.15[b]	—	0.34—1.74	—	2.5—24.5	3.6	0.78	119
	Rabbit skin	0.2 *M* PO_4^{3-}; pH 7.3	31—150	—	—	—	—	1.99—9.65	5.7	0.76	102
	Rabbit skin	—	1—7.2	—	—	—	—	—	0.03	1.20	102
	Umb. cord	0.2 *M* NaCl	11.1—56	17—39.6	—	—	—	—	2.28	0.82	101
	Umb. cord	0.5 *M* NaCl	11.1—56	—	—	—	—	—	3.18	0.78	101
	Umb. cord	0.1 *N* HCl	11.1—56	—	—	—	—	2.9—15.9	2.79	0.76	101
	Rooster comb	0.1 *M* NaCl; pH 8.0	11.7	6.6	—	0.41	—	30	—	—	120
Chondroitin 4-sulfate	Whale cartilage	0.2 *M* NaCl	2.65	2.15	0.11	—	0.56	0.69	—	—	100
	Cartilage	0.2 *M* NaCl + 0.15 M PO_4^{3-}	1.7—4.8	—	—	—	—	0.37—0.91	3.2	0.74	121,125
Chondroitin 6-sulfate	Shark cartilage	0.2 *M* NaCl	1.85—4.37	1.73—2.59	0.22	—	—	0.44—1.40	0.065	1.14[c]	100,105
	Nasal septa[d]	0.2 *M* NaCl	1.22—4.21	1.39—2.43	—	2.9—6.6	—	0.30—0.82	0.050	1.14	117
Dermatan sulfate	Beef lung	0.2 *M* NaCl + 0.2 M PO_4^{3-}	1—1.55	—	—	—	—	—	3.1	0.74	121,125
Keratan sulfate	Cornea	0.2 *M* NaCl	0.87—1.91	1.16—1.72	—	4.1—6.9	0.47—0.55	0.28—0.55	—	—	122
Heparin	Beef lung	0.1 *M* NaCl[e]	0.45—1.56	—	—	—	—	0.094—0.228	0.154	1.00	88
	Beef lung	0.1 *M* NaCl	0.76—1.18	2.13[f]	—	7.45[f]	0.36—0.44	0.12—0.19	0.158	1.00	123
	Various	—	1.2—1.5	2.10—2.38	—	6.29—8.51	0.49—0.65	—	—	—	124

[a] Aqueous solutions with salt concentration shown.
[b] Recalculation by Cleland and Wang[101] gave 12—40.4.
[c] For sample of M.W. <2.06 × 10^4, a value was 1.7.
[d] Mixture of chondroitin 4-sulfate and 6-sulfate.
[e] Also measured at various solvent conditions.
[f] For sample of M.W. 11,800.

1.13, 1.00, and 0.90, respectively. The effect of the dielectric constant of the solvent on the viscosity of heparin was reported by Yuan and Stivala.[89] In the range of dielectric constant 54.7 to 93.2, the $[\eta]_\infty$ values obtained from Equation 3 were shown to increase linearly with the dielectric constant, while parameter B remained constant. However, desulfation of heparin resulted in decrease in both $[\eta]_\infty$ and B. From these results, they suggested that heparin molecules behave as a Gaussian coil when charges are effectively swamped.

Earlier physicochemical studies of hyaluronate in low concentration in dilute solution have characterized[9] the molecule as a *random coil with some stiffness.* The description requires considerable modification in view of the new insights gained from recent hydrodynamic studies and from the application of the technologies of physical biochemistry.

Variation of pH of glycosaminoglycan solutions resulted in changes in viscosity, as expected from changes in the charge density of the polyelectrolyte. Measurements of relative viscosity[90] and intrinsic viscosity[91] of hyaluronate as a function of pH showed sharp decrease in those values with decreasing pH near pH 3.5 to 2.5, which is in accordance with the pK of the carboxyl group. Mathews and Decker[92] reported a rapid drop in viscosity of hyaluronic acid, when the pH of the solution was increased from neutral to 12.5. They explained the result as a suggestion of a reversible conformational change of the hyaluronate molecule resulting from ionization of hydroxyl groups (pK about 12) — perhaps the C-4 hydroxyl groups of the glucosamine residue — and thus destabilization of ordered conformation present in lower pH.

Barrett and Harrington[93] and Barrett[94] reported a tenfold increase in $[\eta]$ of hyaluronate when the pH of the solution was increased from 7.0 to 7.5, and explained the result as cooperative structural transition. They obtained the viscosity results from measurement at low shear-limit (<0.01 sec^{-1}) using a cartesian diver viscometer at 25°C for solutions of 0.25%, 0.125%, and 0.0625% hyaluronate (at pH 7.5, they measured down to 0.00195%) in phosphate buffer of ionic strength 0.1. (This author's group could not observe any significant difference in the viscosity of hyaluronate in the pH range 6 to 8 from measurements on dilute solutions with an Ostwald-type viscometer.) They also showed, in parallel to viscosity, a sharp change in flow-induced birefringence and extinction angle measured over a velocity gradient range of 5 to 100 sec^{-1}. However, no significant variation in sedimentation coefficient and partial molar volume of hyaluronate molecule was reported.

Stivala and Liberti[95] examined the $[\eta]$ of heparin in the pH range of 2 to 7, and reported that $[\eta]$ increases between pH 2.0 and 3.3, beyond which further increase in pH produces no appreciable rise in $[\eta]$. This finding was explained as chain expansion as a result of electrostatic repulsion of the dissociated sulfate groups (there are twice as many sulfate groups as carboxyl in heparin). Lages and Stivala[96] reported intrinsic viscosity and sedimentation coefficient of heparin as a function of Cu^{2+} and Ca^{2+} ion concentration, and suggested that binding of the cations by heparin was achieved without a major alteration in the conformation of a flexible, randomly coiled chain.

Viscosity measurements of chrondroitin 4-sulfate with various added electrolytes including HCl were reported by Mathews[97] and by Eyring and Yang.[98] Mathews[97] showed that the viscosity of the polymer was dependent on the counterion charge and concentration, but not directly on the ionic strength (μ) if the salts were of different types. He explained the independence of μ in terms of high negative potential of the polyanion, the properties of which are little influenced by negative ions. Cleland[99] reported that $[\eta]$ of hyaluronate varied linearly with $M^{-1/2}$ in similar fashion as carboxymethyl cellulose (CMC) of similar $[\eta]$ value. He also showed that the second virial coefficients of the polymers were identical at given ionic strength. Tanaka[100] measured intrinsic

viscosity of sodium and calcium salts of various glycosaminoglycans and showed linear relationships between $[\eta]$ and the inverse of the square root of the salt concentration, which is proportional to ionic strength.

From studies on fractionated hyaluronate, Cleland and Wang[101] showed that $[\eta]$ of the fraction of molecular weight below 10^5 falls below the values calculated from parameters k_m and α obtained from higher molecular weight fractions (see Table 1), possibly due to nongaussian behavior of short chains becoming approximately more nearly a wormlike chain. A similar conclusion was drawn by Shimada and Matsumura,[102] who showed two different lines in plots of $[\eta]$ versus log M for hyaluronate of different molecular weight ranges, 1 to 7.2×10^4 and 0.31 to 1.5×10^6. They also reported that reduced viscosity (η_{sp}/c) was independent of c when $M \leq 2.1 \times 10^4$, whereas higher molecular weight fractions showed lower reduced viscosity values with dilution.

Cleland[103] showed that the double logarithmic plots of the $[\eta] \cdot \overline{M}_w$ ($\overline{M}_w$ is weight average) against degree of polymerization (n) in the range of $\overline{M}_w$ of 10^4 to 2×10^4 permitted a straight-line fit, $\log [\eta] \cdot M_w = 2.07 + 2.0 \cdot \log[2n]_w$, for all available data of glycosaminoglycans, including hyaluronic acid, heparin, and chondroitin sulfate as well as sodium carboxymethyl cellulose. Such general behaviors of different polymers were attributed to a similarity in the short-chain hydrodynamic behavior of the polymers, despite the variation in chain structure and linear charge density. In addition, the results indicated a value of 1.0 for the Mark-Houwink parameter α for the short chains.

Measurements of small-angle X-ray of hyaluronate of high molecular weight were consistently 4 to 6 nm in 0.05 *N* HNO_3, and 4 mm in 0.2 *M* NaCl.[104] Tanaka[105] gave 5.0 and 4.8 nm, respectively, for chondroitin 4-sulfate and 6-sulfate from analysis of sedimentation data based on a wormlike model.

The viscosity of heparin under low shear stress was reported by Chung and Ellerton[106] in water and in the presence of various salts, and at different pHs. With increasing shear rate, $[\eta]$ decreased; the greatest effect was in water, and the effect decreased with increasing salt concentration. MacGregor and Bowness[107] studied viscosity and sedimentation behaviors of sodium and calcium salts of chondroitin 4-sulfate and compared the data with corresponding salts of proteoglycans.

Hallett and Gray[108] utilized a quasielastic light scattering technique to determine the hydrodynamic properties of hyaluronate solutions by measuring the translational diffusion exhibited by polystyrene spheres in the solutions. In the absence of added salt, reduced microscopic viscosity showed maxima near a concentration of 1.0 mg/m*l*, which was related to the expansion of the hyaluronate molecule with dilution.

Balazs[109] showed that hyaluronic acid solutions above a concentration of 3 mg/m*l* with added salt exhibit elastic properties at the pH range 2.0 to 2.7 (especially at 2.5), forming a viscoelastic paste. Such viscoelastic putty formation was reported to be dependent on hyaluronate molecular weight and concentration, and on temperature. Later, Gibbs et al.[110] measured the dynamic viscoelastic properties of the solutions over the frequency range 0.02 to 1.62 at various conditions. The solutions were shown to exhibit a sharp transition from viscous to elastic behavior as the strain frequency increased. This effect was attributed to a stiffening of the hyaluronate molecule. Optical studies by Chakrabarti and Balazs[111] related this viscoelastic property to the formation of crosslinks which might represent ordered array in the polymer chain.

The effects of ethanol on the optical and viscosity properties of hyaluronate at varying ethanol concentration, hyaluronate concentration, pH, and temperature were reported by Park and Chakrabarti.[112-114] Viscosity behaviors are shown in Figure 5.

The results showed reversible conformational transition of the polymer. Even though $[\eta]$ was lower in mixed solvent at pH 2.5 than in water, the reduced viscosity

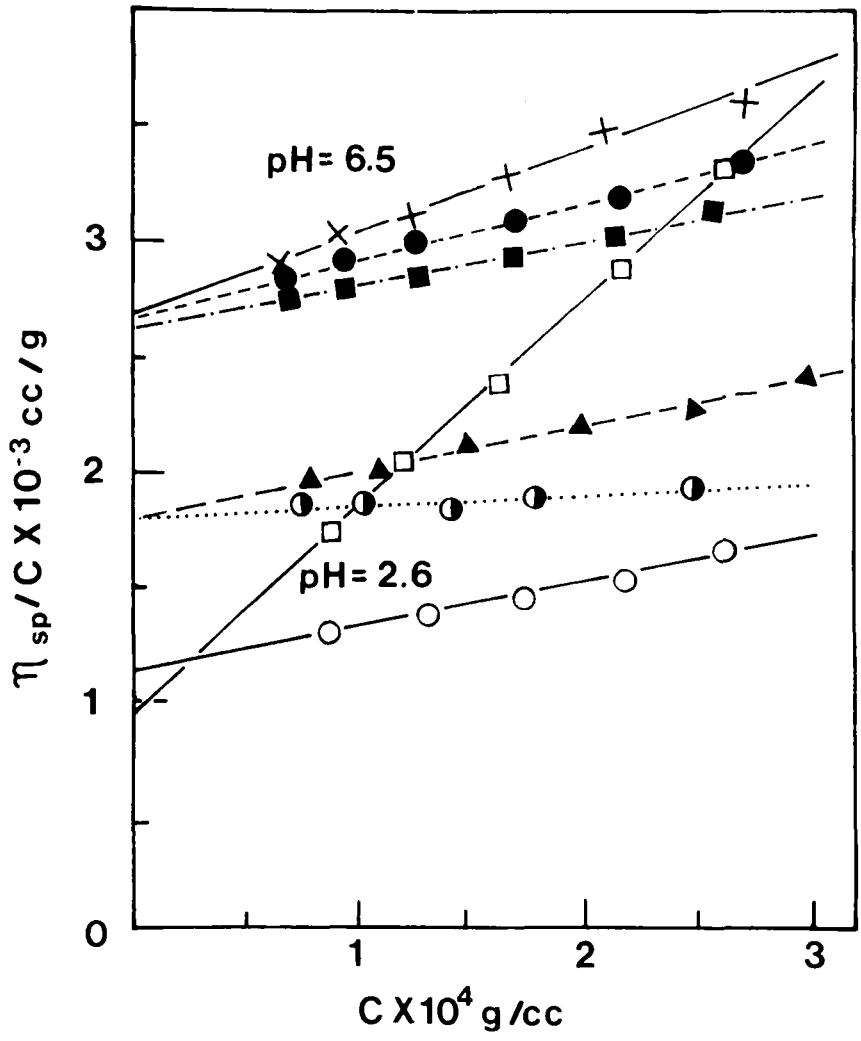


FIGURE 5. Reduced viscosity vs. concentration of hyaluronic acid in various solvent environments at 25°C. 10% ethanol solution at pH 6.5 (X); 1% $HCONH^2$ solution at pH 6.5 (•); aqueous solution at pH 6.5 (■); 10% ethanol solution at pH 2.6 (□); 10% ethanol and 1% $HCONH_2$ solution at pH 2.6 (▲); 1% $HCONH_2$ solution at pH 2.6 (•); aqueous solution at pH 2.6 (o). Differences in intrinsic viscosities $[\eta_{sp}/c]_{c\to 0}$ with pH and solvent condition were attributed to different conformations of hyaluronic acid, and this viewpoint was supported by circular dichroism studies (see section on ORD and CD). (From Park, J. W. and Chakrabarti, B., *Biopolymers*, 17, 1323, 1978. With permission.)

in mixed solvent at low pH increased sharply with hyaluronate concentration, resulting in a gellike state. This was explained by increased intermolecular interaction giving large values of the Huggins constant. Similar viscosity behavior near pH 7 was reported in the presence of Cu^{2+} for hyaluronate[115] and for heparin.[106]

The sedimentation and diffusion values also depend on mol wt and ionic strength. Varga et al.[116] found that both diffusion and sedimentation coefficients of hyaluronate were sensitive to changes in the ionic strength of the solvent, with the dependence most marked as $\mu \to 0$; when μ is lowered, sedimentation gradually decreased, while diffusion increased. A relationship between the sedimentation constant and mol wt for chondroitin sulfate, $s^{\circ}_{20,w} = 2.3 \times 10^{-2}\ M^{0.44}$, was obtained by Wasteson.[117] Cleland

and Wang[101] used Wasteson's data to obtain a relationship for sedimentation and the diffusion constant: log $(s^{\circ}_{20,w})$ = 14.681 − 0.413 log M_{sd}, and log d_o = −3.28 − 0.508 log M_{sd}, in 0.2 *M* NaCl, where M_{sd} is the mol wt based on sedimentation and diffusion data. Tanaka[105] plotted $s^{\circ}_{20,w}$ in 0.2 *M* NaCl against $M^{1/2}$ for chondroitin 4-sulfate and 6-sulfate and obtained straight lines, the intercepts of which were not zero, but definitely positive. From this result, he assumed that the secondary structure of chondroitin sulfates is a semiflexible coil. Shimada and Matsumura[102] showed that l/s did not change linearly with respect to concentration of hyaluronate of mol wt 1.3×10^6 and 1.5×10^6.

Coles and Jennings[118] studied electric-field-induced orientational birefringence of solutions of heparin, hyaluronate, and other biopolymers. They all obeyed Kerr's law; relaxation times of 0.3 msec for hyaluronic acid of 4.4 mg/m*l*, and 0.05 msec for heparin of 44 mg/m*l*, were determined.

X-RAY DIFFRACTION STUDIES

X-ray crystallography remains fundamental to all studies of the conformation of polymer chains. It is beyond the scope of this review to discuss the basic theory of X-ray diffraction in detail; the reader is referred to the texts on this subject. The three-dimensional lattice can be described in terms of the basic repeating unit, the unit cell, and equally spaced sets of planes in the lattice can be defined in terms of their Miller indices, hkl, where h, k, and l are defined as the intersections of the particular set of planes with a, b, and c axes, respectively, of a single unit cell. A typical X-ray diffractogram of an oriented fiber consists of a number of spots, each of which is a result of inflection from different sets of hkl planes. The positions of these spots define the size and shape of the unit cell. The perpendicular distance, known as d-spacing, between the planes that gives rise to a particular reflection is calculated from Bragg's equation:

$$n\lambda = 2d \sin\theta$$

where λ is the wavelength of the radiation, d is the perpendicular distance, and θ is the angle between the planes and the beam. Due to interferences between the waves scattered by successive planes, reflection occurs only when a wave scattered by one plane of a set is an integral number, n, of wavelengths behind the wave scattered by the next plane of the same set.

The major problem associated with deriving a polysaccharide structure is that the number of parameters needed to describe a segment of the chain are far greater than the number of diffraction spots. Thus, it is necessary to start with a model that is reasonable on steric grounds and then, by comparison between observed and calculated diffraction data, to modify the original postulate to bring it to the best possible agreement.

The significant contributions in the mathematical analysis of polysaccharide conformations are those of Jones,[126] who presented the virtual bond method, and Ramachandran,[127] who introduced the ϕ, ψ method. Jones's[126] method requires a knowledge of helix symmetry and pitch. The sugar unit of the polysaccharide is rotated around the virtual bond that joins the linkage atoms. The steric feasibility is calculated by computing the interatomic distances between the residues. Ramachandran's[127] method allows examination of the rotation of contiguous sugar residues around the glycosidic bonds. The method scans the entire possible conformational space available to a polymer; it has been extensively used for the conformational analysis of polysaccharides.[128-137]

Considerable progress was made when Arnott and Scott[138] introduced the method

of linked atom, least square analysis. The method allows the generation of a certain degree of flexibility in the ring geometry of a chain. The atomic coordinates are calculated from the input data such as the average bond lengths, bond angles, torsion angles, and their standard deviation, as derived from the crystal structure results of small molecules. The geometric parameters are varied, within the limits of their standard deviations, to take into account the constraints, such as the ring closure, the repeat distance along the chain, and hydrogen bond criteria. Simultaneously, another new method[139] became available for studying the packing of the chains in the crystal lattice. In this method of calculating the molecular position in a lattice of known dimensions, the repulsive energy is approximated by a sum of quadratic nonbonded interatomic potential functions and the lattice energy sum is minimized by full-matrix least squares. The calculated packing models are sufficiently accurate to serve as a starting point for structure factor least squares refinement based on diffraction data. The successful application of these two procedures improved the precision in the conformational analysis of polysaccharides.[140-142]

Bettelheim[143] (1959) demonstrated that it was possible to obtain ordered fibers of glycosaminoglycans for X-ray diffraction measurements. In 1972, Atkins and Sheehan[144] published X-ray diffractograms of hyaluronate fiber, which indicated that the molecule could be described as a linear helix. Since then, X-ray diffractograms have been recorded[145-169] for oriented fibers of each glycosaminoglycan within a surprisingly short time. Glycosaminoglycans can be dry-spun into fibers or cast into films which can be oriented further, and sometimes also crystallized, by application of constant tension, at appropriate temperature and relative humidity. These ordered specimens permit X-ray diffraction studies, which lead in favorable cases (see next section on hyaluronic acid) to models depicting all the interatomic interactions within and between the molecules.

Except for hyaluronic acid[159-161] and chondroitin 4-sulfate,[168] detailed X-ray studies of glycosaminoglycans are still lacking. Nevertheless, the fact that emerges from these studies is that all these molecules show some sort of helical configurations. The helix symmetries and average axial periodicities (h) per disaccharide unit of glycosaminoglycans are given in Table 3. (In the following sections each polysaccharide will be discussed in detail.)

To the extent that these polysaccharides are related in covalent structure, they might be expected to show some similarity in their conformations, interactions, and biological functions. Indeed, it is evident from Table 3 that the chemical similarities among the glycosaminoglycans (for instance, polysaccharides with alternating 1 → 3 and 1 → 4 glycosidic linkages) result in conformational similarities. For example, twofold helices are observed for hyaluronate, the chondroitin sulfates, dermatan sulfate, and keratan sulfate; except for keratan sulfate, all can exist also as threefold helices. Although the linkages seem to be dominant conformational determinants, the substituent groups in the sugar rings may be no less important, as indicated by keratan sulfate (no uronic acid moiety) and hyaluronate (no sulfate): Keratan sulfate has a twofold helix as its only form whereas sodium hyaluronate has a fourfold helix with contracted axial translation per residue (h = 0.85 nm) as its dominant form.

Hyaluronic Acid

Preliminary models for hyaluronate backbone conformations were set up by means of a computer search.[131] A preference for a left-handed helical strcture was found. The helical structure of hyaluronate and its acid form were first reported by Atkins and co-workers;[144,146] they suggested a left-handed threefold helix for hyaluronate and a twofold structure for the free-acid form with a rise per disaccharide on the helix axis of 0.98 nm. Two hexagonal forms, both with the fiber axis, c = 2.85 nm, for hyalu-

TABLE 3

Helix Symmetries and Average Axial Periodicities (h, nm) per Disaccharide for the Polysaccharide Chains of Glycosaminoglycans[a]

	h values[b] of glycosaminoglycans						
Helix symmetry[c]	HA	KS	Ch 6-S	Ch 4-S	DS[d]	HP	HPS
Twofold[e]	0.98[h,i,j]	0.945[k]	0.93[l]	0.98[m]	0.96[n] 0.97[o]	0.80—0.87[p,n]	0.93[q,r,s]
Threefold[f]	0.95 [h,i,n] 0.94[v]	—	0.95[t] 0.96[w]	0.96[m,q,s,x] 0.98[s] 0.913*	0.95[o,n]	—	—
Fourfold	0.85[i,v,**] 0.93g*** 0.82†	—	—	—	—	—	—
Eighthfold	—	—	0.98[w]	—	0.93[o,n]	—	—

[a] This is a revised form of the table given in Reference 164; HA, hyaluronic acid; KS, keratan sulfate; Ch 6-S, chondroitin 6-sulfate; Ch 4-S, chondroitin 4-sulfate; DS, dermatan sulfate; HP, heparin; HPS, heparan sulfate. Most of the data were recorded for Na salt — for details see text.

[b] h values are dependent on relative humidity and hence are not always strictly as shown.

[c] The number of disaccharide residues per pitch length is indicated. For details of helix symmetry, see Reference 164.

[d] The apparent similarities between dermatan sulfate and chondroitin sulfate helixes indicate that the iduronates in DS have the same C-1 chair conformation as glucuronate; however, the dissimilarity in h values of eightfold helices of DS and Ch 6-S do not rule out the possibility of 1-C chair conformation. The large difference in values between DS and HP in their twofold helices certainly indicate that the iduronate conformation in DS is different from that in HP (for details see text).

[e] The twofold helixes for hyaluronate, Ch 6-S, and Ch 4-S have been obtained only under acidic condition.

[f] It has been claimed[164] that the threefold helix of hyaluronates can be obtained only for calcium hyaluronates or for the calcium-contaminated sodium salt of hyaluronic acid.

[g] The longer periodicity was obtained only in the presence of 10% Ch 6-S.

[h] Reference 144.

[i] Reference 147.

[j] Reference 148.

[k] Reference 156.

[l] Reference 145.

[m] Reference 149.

[n] Reference 153.

[o] Reference 150.

[p] Reference 152.

[q] Reference 151.

[r] Reference 155.

[s] Reference 166.

[t] Reference 146.

[u] Reference 161.

[v] Reference 162.

[w] Reference 154.

[x] Reference 168.

[y] Reference 169.

[z] Reference 36.

[α] Reference 167.

** Reference 159.

*** Reference 164.

† Reference 163.

ronate were also suggested. A year later, the double helical model for both hyaluronate and hyaluronic acid was proposed.[147,148] The unit cell in this model was observed to be orthorhombic with the fiber axis of 3.37 nm. The proposed double helix was depicted as two identical, left-handed strands which are antiparallel to each other. Each strand has four disaccharide residues (fourfold) per pitch length. After more careful consideration based upon detailed X-ray structure-factor calculations, this model was rejected and replaced with a contracted single-stranded model.[159]

Detailed X-ray diffraction studies of hyaluronate show that in a condensed phase the polyanion can adopt a conformation belonging to one of two widely differing classes, which were termed compressed and extended.[159-161] These two classes differ principally in the average axial repeat per disaccharide (h), with h = 0.85 nm being typical of the comppressed form and h = 0.95 nm typical of the extended form. The extended hyaluronates can assume a variety of conformations[146,147,161] with different screw symmetries involving turn angles per disaccharide, t, of −90°, −120°, or 180°, but with the value of h remaining relatively constant. In the compressed form,[159] t is close to −90°. The negative sign indicates the left-handed chirality. Comparison of refined structures for members of each class indicates that the different h values arise from alterations in the two sets of linkage conformation angles rather than from some dramatic alteration of sugar ring geometry. The intermolecular, rather than intramolecular, forces should, therefore, be responsible for the existence of more than one crystalline phase.[162]

Guss et al.[159] first reported the detailed structures of two variants of the compact polymorph of sodium hyaluronate. One, the tetragonal form, has a = b = 0.989 nm and c = 3.394, in which two regular fourfold helical polysaccharide chains pass through each unit cell. The unit cell thus contains eight disaccharide residues, eight coordinated sodium ions, and no water molecules (Figure 6). The chains are antiparallel to each other, and each is a left-handed helix. At higher relative humidity, the second variant (orthorhombic), very similar to the tetragonal form, but different in a dimension of the lattice, was observed (a = 1.153 nm). In this form the hyaluronate helix is twofold. Both structures are stabilized intramolecularly by hydrogen bonds between the C-4 hydroxyl group of *N*-acetylglucosamine and the ring oxygen of glucuronic acid across the 1→3 glycosidic linkage and by N-H...O (carboxyl oxygen) hydrogen bonds across the 1 → 4 linkages, and intermolecularly by a network of hydrogen bonds and ONa^+...O bridges.

Winter et al.[161] later reported the trigonal crystalline form of sodium hyaluronate. In this conformation, the molecule is highly extended with an axial advance per disaccharide of 0.95 nm. The polysaccharide chain in this structure is a regular, left-handed threefold helix and to a certain extent similar to the previously proposed[144,146] form.

The major difference between four- and threefold helices in the side chain orientation involves a shift from an approximately gauche-trans rotational isomer for the former to the gauche-gauche conformation for the latter structure. Both exhibit left-handed chirality and the chains are packed in an antiparallel manner and stabilized by an extensive network of intramolecular and intermolecular hydrogen bonds and O...Na^+...O bridges. However, the intramolecular hydrogen bond formations are not the same in both structures; N−H...O hydrogen bonds of the fourfold form are absent in the threefold form; instead it has hydrogen bonds between the C-3 hydroxyl group of glucuronic acid and the ring oxygen of *N*-acetylglucosamine. A similar threefold helix was proposed[162] for the calcium salt of hyaluronate, where adjacent antiparallel chains are tied together through a COO^-...Ca^{2+}...^-OOC bridge, and the coordination of each Ca^{2+} is completed by three pairs of dyadically related water molecules (Figure 7). As a matter of fact, calcium ion impurities, which may be responsible for the threefold helix, were suspected in their earlier trigonal form[161] of sodium hyaluronate. These

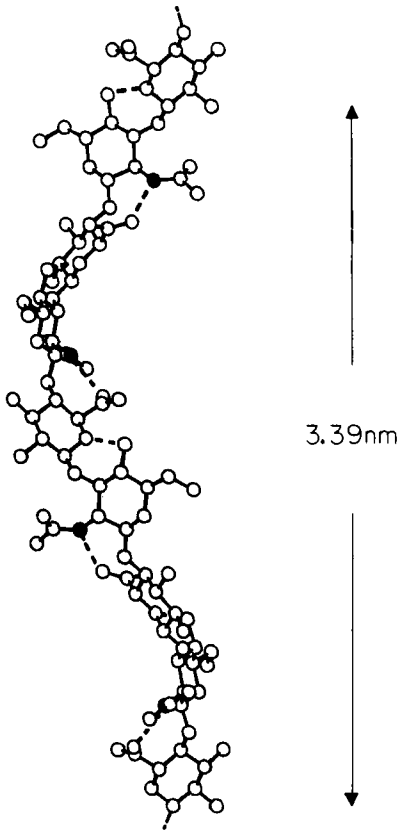


FIGURE 6A. A single hyaluronate chain from the tetragonal structure. The hydrogen bands N^{B}—H...$O^{A}_{(6a)}$ of 0.278 nm and $O^{B}_{(4)}$—H...$O^{A}_{(5)}$ of 0.253 nm are shown. (Superscripts A and B are *N*-acetylglucosamine and glucuronic acid, respectively; $O^{B}_{(4)}$ is C-4 hydroxyl oxygen; $OA_{(5)}$ and $O^{A}_{(6a)}$ are ring oxygen and carboxyl oxygen, respectively.) Solid circles are nitrogen atoms. (From Guss, J. M., Hukins, D. W. L., Smith, P. J. C., Winter, W. T., Arnott, S., Moorhouse, R., and Rees, D. A., *J. Mol. Biol.*, 95, 359, 1975. With permission. Copyright by Academic Press Inc. (London) Ltd.)

authors further emphasized that the compressed fourfold conformers of hyaluronate[159] cannot participate in this type of divalent cation binding.

The compressed and extended forms also differ between themselves in other aspects. There are extensive interdigitations in the compressed form, where each chain interacts not only with its nearest antiparallel neighbors, but also with its nearest parallel neighbors — eight molecules in all. In contrast, in the extended form one molecule interacts with only three antiparallelneighbors, and there is only one intermolecular hydrogen bond per disaccharide and no interdigitation.

More recently, a left-handed antiparallel double-helical structure has been proposed[163] for hyaluronic acid in the presence of the cations K^+, NH_4^+, Rb^+, and Cs^+. The diffraction pattern attributed to the double helix was obtained in the presence of

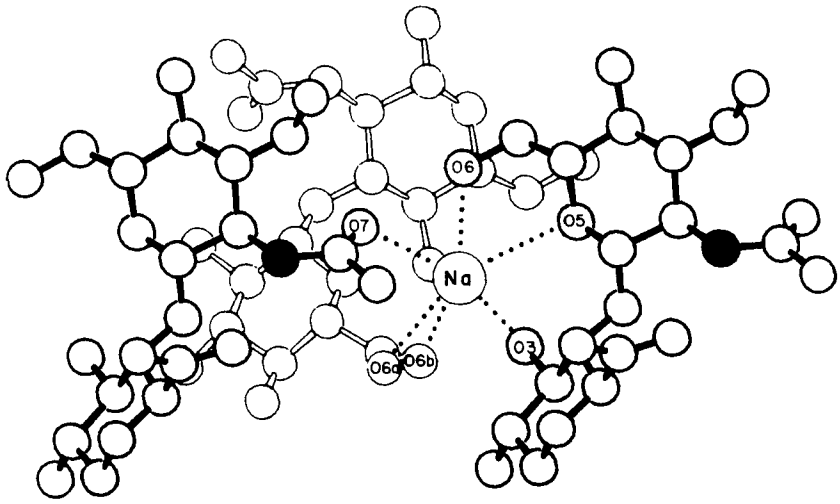


FIGURE 6B. Details of sodium ion environment in the tetragonal structure. Solid circles are nitrogen atoms. View along (100) direction. The center chain (open bonds) is translated a/2 behind the corner chains. Only the contacts to the sodium ion which binds to all three chains in this figure are shown. (From Guss, J. M., Hukins, D. W. L., Smith, P. J. C., Winter, W. T., Arnott, S., Moorhouse, R., and Rees, D. A., *J. Mol. Biol.*, 95, 359, 1975. With permission. Copyright by Academic Press Inc. (London) Ltd.)

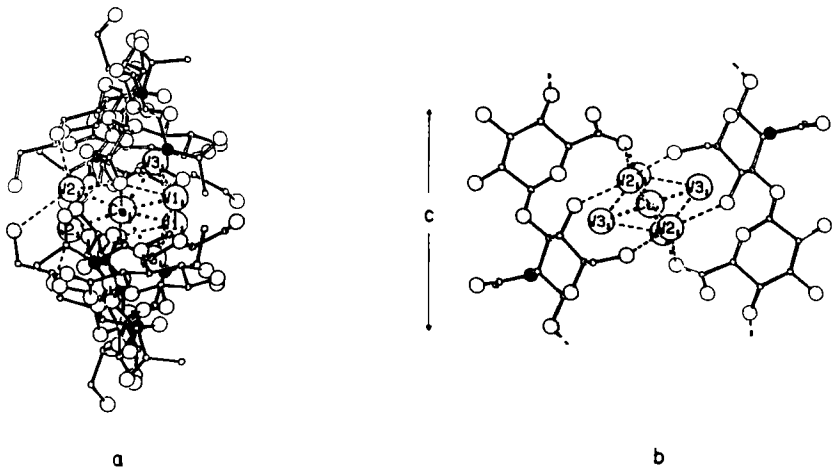


FIGURE 7. Coordination of Ca^{2+} ion by 2 hyaluronate polyanions (threefold helices) and 6 water molecules (W) forming a distorted antiprism viewed (a) parallel and (b) perpendicular to the c axis. The water molecules are extensively hydrogen bonded to the polyanions. (From Winter, W. T. and Arnott, S., *J. Mol. Biol.*, 117, 761, 1977. With permission. Copyright by Academic Press Inc. (London) Ltd.)

potassium ion, only below pH 4.0. However, dialysis of the acid salt of hyaluronic acid against a solution of 1.0 *M* NH_4Cl, RbCl, and CsCl yielded isomorphous diffraction patterns and, more importantly, the phase is produced in the presence of NH_4^+ and Rb^+ without going through the acid form. The features of the proposed structure

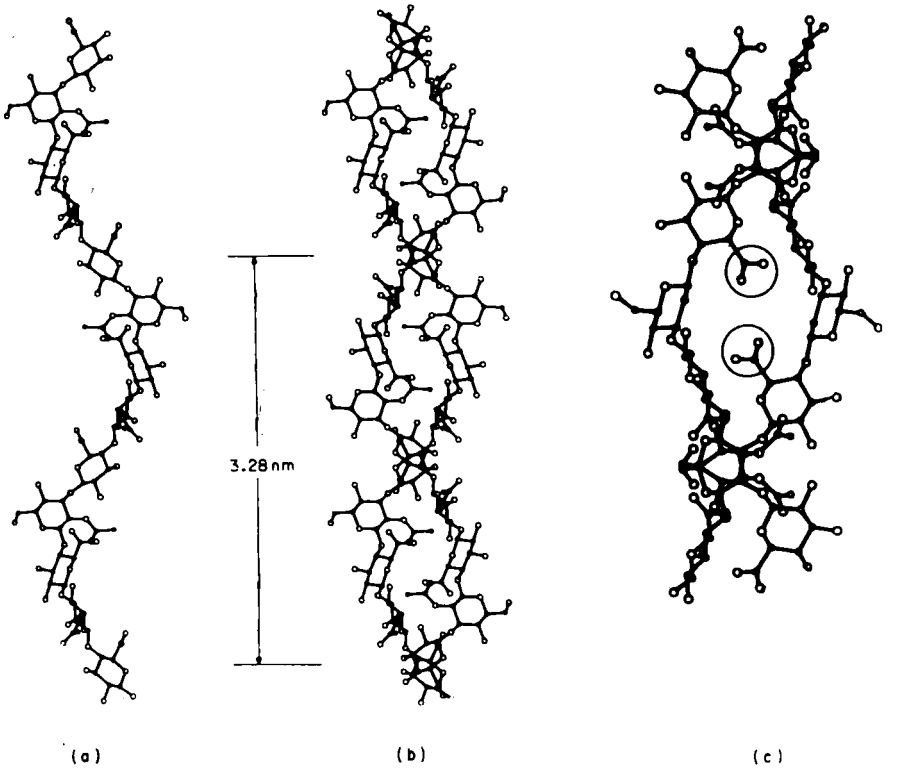


FIGURE 8. The refined molecular conformation for the hyaluronate chain. (a) Conformation of a single chain. (b) Conformation of the double helix viewed along the (100) direction. (c) View of a double helix segment showing the proximity of the carboxyl groups (circled) to the helix axis; this view is along the (110) direction. For the sake of clarity the *N*-acetyl groups are not shown. (From Sheehan, J. K., Gardner, K. H., and Adkins, E. D. T., *J. Mol. Biol.*, 117, 113, 1977. With permission. Copyright by Academic Press Inc. (London) Ltd.)

(Figure 8) are that each chain forms a contracted fourfold helix which intertwines with a neighboring chain of opposite polarity around a common axis, and the presence of hydrophobic and hydrophilic pockets between adjacent double helices. Although the model is consistent with other published hyaluronate structure refinements,[159-162] it does not show any obvious interactions between chains in the double helix that would help stabilize the structure.

Keratan Sulfate

X-ray diffraction patterns of a stretched film of keratan sulfate isolated from bovine cornea were explained as arising from twofold single helices with an axial rise per disaccharide residue of 0.945 nm, which results in an extended chain conformation fringed with charged sulfate side groups[156] (Figure 9).

Computer methods were used to construct a model with the observed symmetry and axial rise per disaccharide residue, with standard bond lengths, bond angles, and pyranose ring conformations, and with a hydrogen bond across the 1 → 4 linkage between the C-3 hydroxyl group of *N*-acetylglucosamine and the ring oxygen of the galactose residue. Stability of the isolated keratan sulfate chain of the extended conformation of this type was questioned,[156] because there seems no possible mechanism by which favorable intramolecular energy terms can accumulate cooperatively to offset the entropy loss associated with conformational ordering. However, the polysaccharide

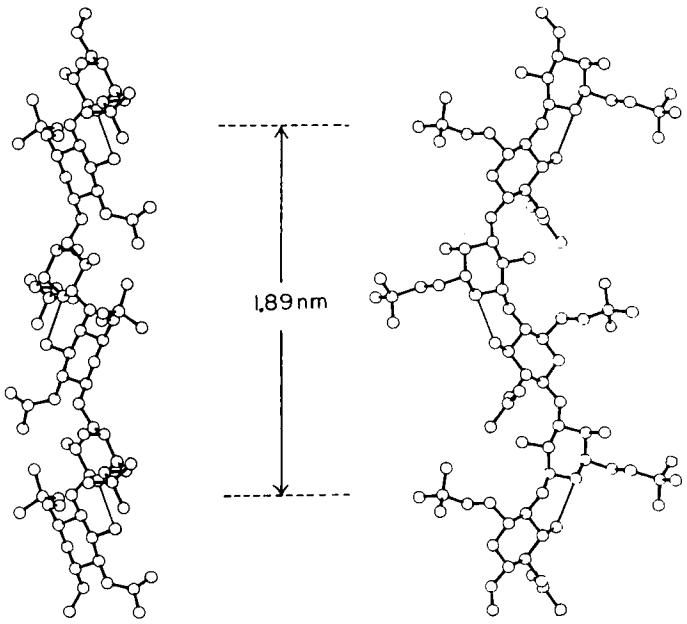


FIGURE 9. Two views of the twofold helical model for keratan sulfate. Both are projections perpendicular to the helix axis; they are related to each other by a 45° rotation about it. (From Arnott, S., Guss, J. M., Hukins, D. W. L., Dea, I. C. M., and Rees, D. A., *J. Mol. Biol.*, 88, 175, 1974. With permission. Copyright by Academic Press Inc. (London) Ltd.)

chain could exist in extended form in the intercellular matrix where cooperative stabilization might be derived from intermolecular contact. Because of the heterogeneity[82] in the sulfate group content per disaccharide residue, Arnott et al.[156] did not rule out the possibility of different conformational behavior of the molecule due to variable charge density.

Chondroitin 6-Sulfate

Earlier investigation[145] on X-ray fiber diffraction patterns from chondroitin 6-sulfate (Ch 6-S) indicated a threefold helical structure in the sodium salt of the polyanion and twofold screw symmetry in the acid form. The same study claimed that although the threefold helical structure of Ch 6-S had apparent similarity with that of sodium hyaluronate,[144,146] these helices did not pack in a hexagonal manner as did hyaluronate. This observation led these workers to suggest that either the sulfate groups would dictate the interchain packing scheme or the coordination environment of the two sodium ions in Ch 6-S per disaccharide repeat might influence the chain packing behavior. Sodium salts of chondroitin 6-sulfate and some further sulfated derivatives of the polymer were later studied by Arnott et al.[153] They reported two ordered structures, three- and eightfold helices, where two chains pack parallel to one another in regular arrays. Both structures contain single helices (Figure 10) with almost identical projected disaccharide lengths (0.96 and 0.98 nm), but very different turn angles between successive disaccharides (120° for a threefold and 45° for an eightfold helix). These authors further indicated that the azimuthal distribution of charged side groups is very different in the two conformations, which leads to changes in glycosidic conformational angles. These changes have little effect on axial periodicities per disaccharide residue.

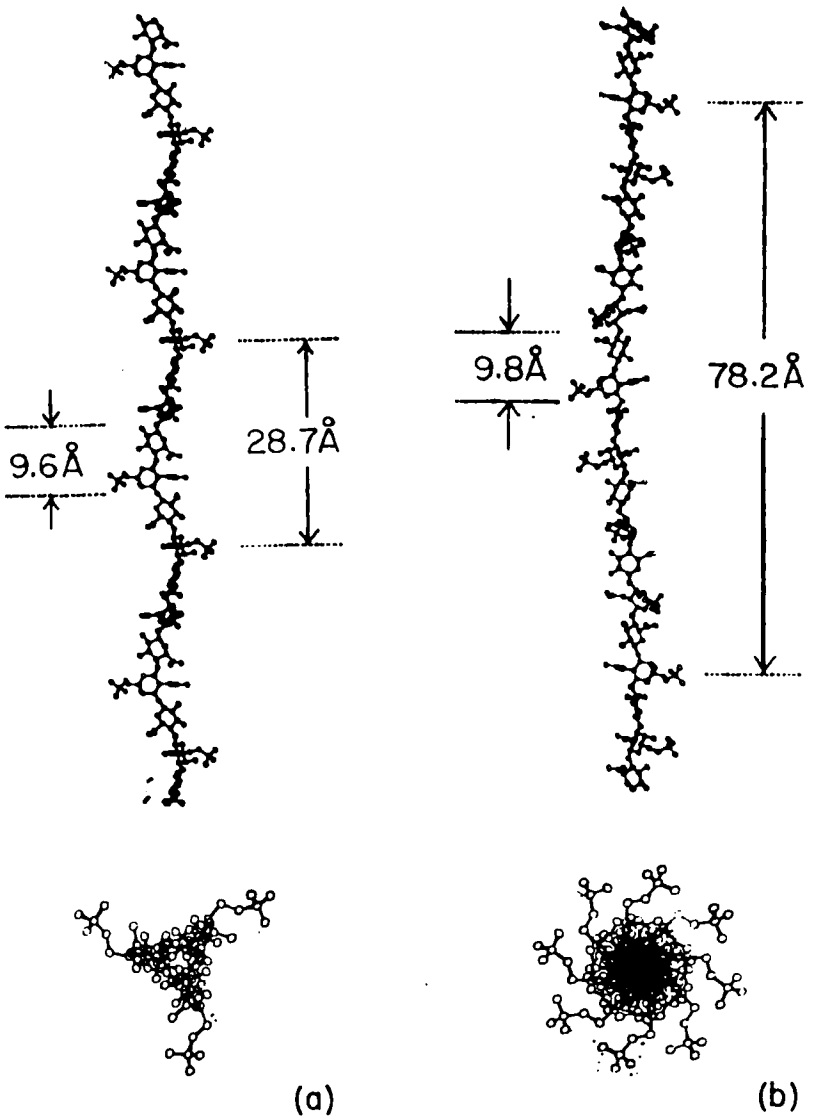


FIGURE 10. Projections of models for the helical molecules (viewed both parallel and perpendicular to the helix axes) for (a) the single threefold helix of chondroitin 6-sulfate, and (b) the single eightfold helix of chondroitin 6-sulfate. Chains are shown as left-handed. (From Arnott, S., Guss, J. M., Hukins, D. W. L., and Mathews, M. B., *Science*, 180, 743, 1973. With permission. Copyright 1973 by the American Association for the Advancement of Science.)

Chondroitin 4-Sulfate

Bettelheim[36] obtained partially oriented films of the calcium salt of chondroitin 4-sulfate and observed meridional arcs at spacings 0.98 and 0.327 nm, which were assigned as first and third orders of the identity period. Atkins and co-workers[149,151] reported ordered conformation of both the sodium-salt and free-acid forms of chondroitin 4-sulfate from their X-ray diffraction studies of oriented films. The X-ray pattern of sodium chondroitin 4-sulfate, similar to those found for the sodium salt of Ch 6-S[145] and for sodium hyaluronate,[144] was interpreted as a threefold helical structure with the repeat distance of 2.85 nm and the projected height of 0.95 nm. In acidic pH, conformation of Ch 4-S reportedly changes to a twofold helix.[149] The free-acid form

was obtained by immersion of crystalline specimens of the sodium salt in acidic ethanol bath. X-ray results showed a decreased layer line spacing of 1.96 nm with meridional reflexions on even layer lines only, and the unit cell was orthorhombic.[149]

The conformation of the calcium salt of Ch 4-S was further examined[167] by X-ray analysis and the results were not significantly different from those of the sodium salt.[149] The repeat length per disaccharide was 0.913 nm and the molecular chain had threefold screw symmetry. The value of 0.913 nm is significantly different from 0.980 nm, which was obtained by Bettelheim.[36] The difference was attributed[167] to different methods of specimen preparation.

Atkins et al.[157] reported that aggregated chondroitin 4-sulfate-protein complex, disaggregated proteoglycan, and reconstituted disaggregated proteoglycan gave similar X-ray photographs which could be correlated with the Ch 4-S component. The patterns were interpreted as twofold helical conformations, similar to that observed previously for the free-acid form of the polymer. The presence of protein, keratan sulfate, and hyaluronic acid in the aggregate proteoglycan did not significantly alter the conformation of Ch 4-S.

The detailed three-dimensional structural analysis of chondroitin 4-sulfate is now available,[168] in which the crystal structure and molecular conformation of a regular threefold helical form of the polymer have been refined. The polyanion conformation is stabilized by an intramolecular hydrogen bond between O(3) of glucuronic acid and the ring oxygen of hexosamine across a β (1 $\rightarrow$ 4) linkage. Across the 1 $\rightarrow$ 3 linkage, there is no hydrogen bond. Packing of Ch 4-S is stabilized by the participation, once as a donor and once as an acceptor, of each disaccharide in two intermolecular hydrogen bonds involving O(2) of glucuronate and O(7) of hexosamine (Figure 11). This hydrogen bonding scheme has not been observed in either of the threefold hyaluronate crystal structures.[161,162]

Diffractograms have recently been obtained from sodium and calcium salts of a proteoglycan rich in Ch 4-S isolated from swarm rat chondrosarcoma.[169] When sodium is the only countercation associated with the proteoglycan, the oriented polysaccharide chains were found to assume a threefold helical conformation which changes into a twofold structure upon addition of small amounts of calcium salt. The calcium ion-induced helix is stabilized intramolecularly by $OSO_3^-...Ca^{2+}...^-OOC$ coordination across the β (1 $\rightarrow$ 3) linkage in addition to the reported[168] hydrogen bond across β (1 $\rightarrow$ 4) for the threefold helical structure. Within the lattice, adjacent parallel chains interact through $^-COO...Ca^{2+}...OOC^-$ bridges, and each calcium coordination shell is completed by 5 molecules of water.

Dermatan Sulfate

Dermatan sulfate differs from chondroitin 4-sulfate in that it contains α-L-iduronic acid rather than β-D-glucuronic acid. Heparin and heparan sulfate also contain a certain percentage of L-iduronate in their repeating disaccharide unit. The major question that has arisen concerns the ring conformation associated with this uronic acid moiety. It is believed that the conformational and configurational differences of the uronic acid moiety may account for many of the chemical activities and biological functions of these macromolecules. After a preliminary report,[151] detailed X-ray results of dermatan sulfate were published simultaneously by two independent groups of investigators.[150,153] Results were very similar; diffraction patterns show that the molecule in oriented crystalline films occurs as two-, three-, or eightfold helices. The axial rise per disaccharide residue for twofold is 0.97, for threefold 0.95, and for eightfold 0.93 nm. Three types of X-ray diffraction patterns resulted from three different lattices: orthothombic, trigonal, and tetragonal, which contain corresponding helical molecules of

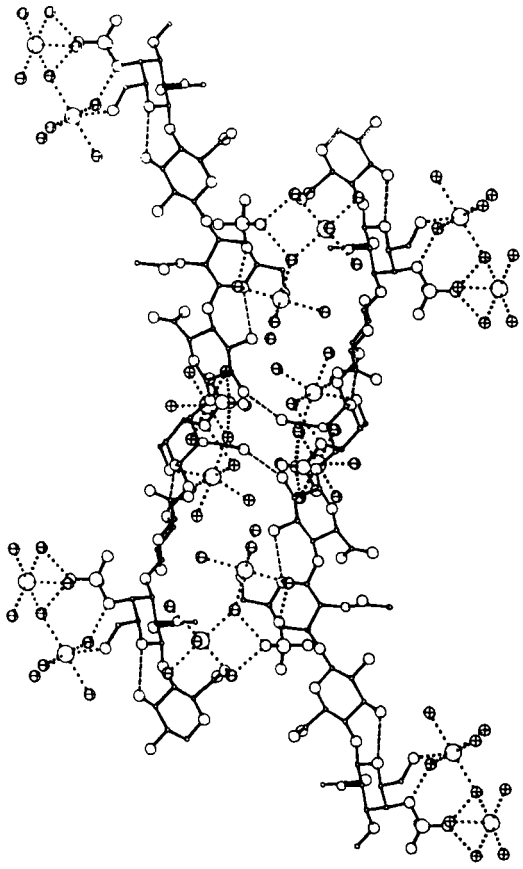


FIGURE 11. Threefold helical form of chondroitin 4-sulfate. Projection along the short diagonal, (001) direction, of the base plane showing interactions between an adjacent pair of polyanions. Sodium ions are the largest circles. Water molecules are circles with crosses. (From Winter, W. T., Arnott, S., Issac, D. H., and Atkins, E. D. T., *J. Mol. Biol.*, 125, 1, 1978. With permission. Copyright by Academic Press Inc. (London) Ltd.)

twofold, threefold, and eightfold symmetries.[153] The twofold helices are similar to those observed for Ch 6-S and hyaluronic acid.

The L-iduronic acid is the C-5 epimer of D-glucuronic acid, so that in the normal C-1 chair form the carboxyl group at C-5 is axially positioned. It is of interest to consider in which of these two chain forms the L-iduronic acid of dermatan sulfate exists. In the alternative 1-C chair form the carboxyl group is positioned equatorially and the glycosidic linkages become diaxial. Fransson[170] has reviewed the evidence for and against particular chair forms of L-iduronic acid.

D-glucuronic acid and L-iduronic acid in the normal C-1 chair form are shown in Figure 12. Such a chair conformation for D-glucuronic acid appears quite satisfactory — all the side groups are equatorially positioned, but the carboxyl group of L-iduronic acid is axially positioned. As shown in the figure, the equatorial position of this group in the alternate 1-C chair conformation radically changes the general shape of the polysaccharides.

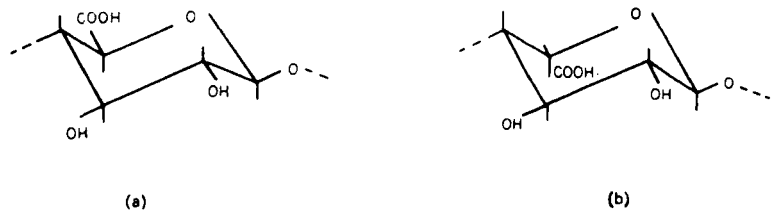


FIGURE 12A. Schematic representation of both uronic acid moieties in the C-1 chair conformation. (a) D-Glucuronic acid. (b) the C_5 epimer L-iduronic acid. The glycosidic linkages are shown by the broken lines. (From Atkins, E. D. T. and Issac, D. H., *J. Mol. Biol.*, 80, 773, 1973. With permission. Copyright by Academic Press Inc. (London) Ltd.)

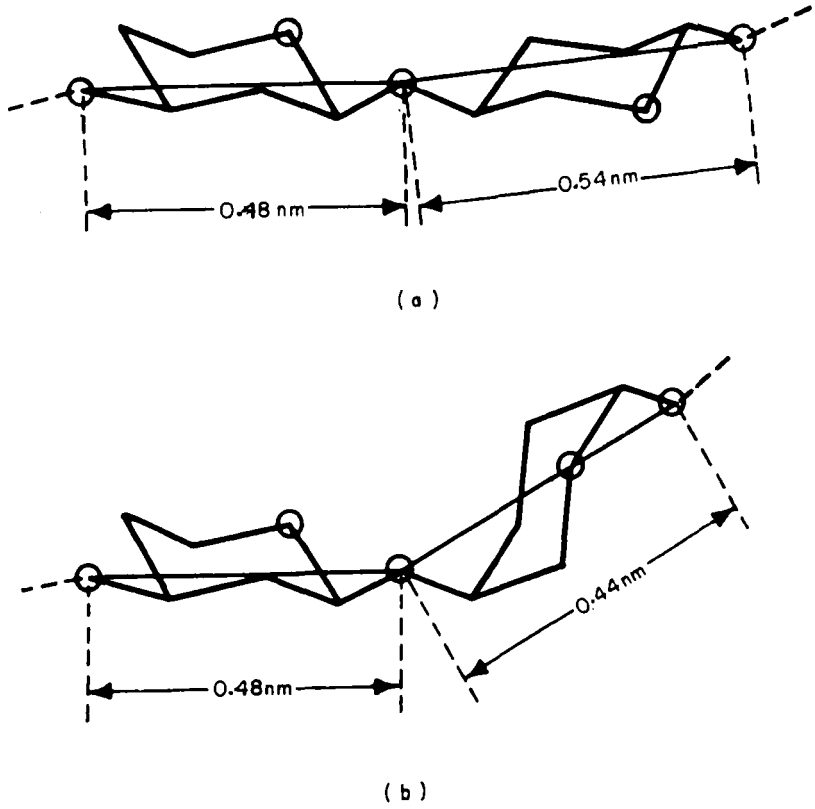


FIGURE 12B. Shape and dimensions of the disaccharide repeat. (a) The uronic acid is in the C-1 chair conformation and the maximum theoretical extension is 0.48 + 0.54 = 1.02 nm. (b) The uronic acid is in the 1-C chair conformation and the maximum theoretical extension is 0.48 + 0.44 = 0.92 nm. Note how the general shape of the covalent repeat changes. (From Atkins, E. D. T. and Issac, D. H., *J. Mol. Biol.*, 80, 773, 1973. With permission. Copyright by Academic Press Inc. (London) Ltd.)

In view of the fact that dermatan sulfate differs significantly in its interaction properties — and probably in its biological functions — from the other members of the group, including heparin which contains L-iduronic acid as the major component, the

conformation of the uronic acid moiety in the polymer became an interesting area of investigation.[42,171-176] Proton NMR studies of dermatan sulfate in solution above 70°C show that the L-iduronate is in the 1-C chair conformation.[177] The rationalization for the conformational specificity from X-ray diffraction results is straightforward. In the preliminary examination of the X-ray results, Atkins and Laurent[151] ruled out the 1-C conformation on the grounds that the maximum projected disaccharide repeat distance, h, that is theoretically possible for dermatan sulfate in the 1-C chair form is 0.92 nm (when stereochemically feasible molecular models are considered, the value decreases to 0.90 nm), which is less than the experimentally observed value of 0.93 nm. On the same argument, Arnott et al.[153] rejected 1-C chairs in the two- and threefold helical forms since he obtained the experimental values of 0.95 and 0.96 nm for these conformers, respectively. However, in the eightfold model, with the value of h only 0.01 greater than 0.92, a fully extended dermatan sulfate helix can be built containing 1-C α = L-iduronate chairs if the standard pyranose ring shape is changed trivially.[153] A detailed analysis of the diffraction is obviously needed for the alternative conformation.

Heparin and Heparan Sulfate

X-ray diffraction studies of heparin and heparan sulfate are still in the preliminary model-building stage. Despite many structural investigations, the covalent chemical repeating unit in heparin and heparan sulfate remains in some doubt. The sulfate content of heparin varies between 4 and 6 sulfate per tetrasaccharide unit. Since the relative amounts of the two uronic acids, D-glucuronic and L-iduronic acid, in both compounds have not yet been ascertained, it is pertinent to ask whether a unique covalent repeating sequence exists for these relatively short molecules. In the preliminary study, it was reported[152] that the sodium salt of heparin crystallizes in a triclinic unit-cell with a measured molecular repeat of 1.59 nm. A more precise X-ray fiber-diffraction of the sodium salt of macromolecular heparin prepared from rat skin was obtained,[158] and the molecular repeat distance was measured as 1.65 nm at 84% relative humidity. At 78% relative humidity, however, the pattern was indexed on an orthorhombic unit cell with a layer-line spacing of 1.73 nm. The noteworthy features emerging from these studies were: (1) both the crystalline modifications involve a tetrasaccharide crystallographic repeat; (2) the high-molecular-weight rat skin heparin can adopt two distinct conformations with different periodicities, whereas pig intestinal mucosa heparin, a low-molecular-weight polymer, shows only 1.65 nm periodicities. Atkins et al.[158] implied that conformational changes due to depolymerization could account for the marked differences between the biological activity of intact and degraded rat skin heparin.

The molecular shapes thus far obtained for heparin from model building can be represented by a twofold helical conformation with the axially projected disaccharide repeat, h, lying in the range of 0.80 to 0.87 nm. The values of h thus obtained were useful enough to suggest the probable conformation of L-iduronate in heparin.[165] The NMR results[177] favor the 1-C chair conformation for this residue. The maximum theoretical extension of such a repeat based on standard atomic coordinates is 0.89 nm, and would be compatible with the observed values of h in the range of 0.80 to 0.87 nm, whereas for the C-1 conformation the theoretical extension maximum is 0.99 nm. This was tested[165] further with the values of the residual index (R) between calculated and observed structure factors and the corresponding chair packing results for the four trial models (twofold helices) involving 1-C and C-1 chairs for α-L-iduronate residue in heparin. The test favors the 1-C conformation. The 1-C chair form is able to form a stabilizing intramolecular hydrogen bond between O(3)-H of the glucosamine residue

and the ring oxygen of the iduronate residue. The other conformation appears to have no obvious molecular hydrogen-bonding possibilities.

Diffraction patterns obtained from the sodium salt of heparan sulfate show Bragg's reflection on the meridian of alternate layer lines. Their spacing is 0.93 nm; the spacing of the layer lines is 1.86 nm,[151,155] indicating a regular twofold helical structure. It was suggested[151] that the linkage between the *N*-acetylglucosamine and glucuronic acid residues is alternately α-D(1→4) and β-D(1→4). In contrast to heparin, the majority of the uronic acid component in heparan sulfate is β-D-glucuronic acid. One can consider three models with regular twofold helixes in which all the linkages are 1 → 4, the hexosamine is in the C-1 chair conformation, and the uronic acid is glucuronate and iduronate that are both C-1, or glucuronate and iduronate that are both 1-C, or only glucuronate in the C-1 conformation. The first possibility is straightforward for production of twofold helixes incorporating standard bond lengths, bond angles, and ring conformation. In this model, the ring oxygen of uronate can be hydrogen bonded to O(3) of hexosamine, stabilizing the conformation. With the other two models, the maximum values of h obtainable are 0.85 and 0.73 nm, respectively, considerably shorter than the observed value of 0.93 nm. A different X-ray fiber diffraction pattern has been obtained for the calcium salt of heparan sulfate, with the value of h dropping to 0.84 nm. The reduced h would favor the model with both glucuronate and iduronate in 1-C chair conformation.[164] It is expected that refinement of the X-ray data will enable the correct model to be established. A more detailed study of the molecular architecture of these biopolymers will undoubtedly reveal much information pertinent to both the biological function and the chemical properties of this important group of polysaccharides.

SPECTROSCOPIC STUDIES

The review in this section is concentrated on those spectroscopic techniques that have been used successfully for determining the structure of glycosaminoglycans in solution. The optical rotatory dispersion (ORD) and circular dichroism (CD) properties of these polymers have been extensively studied. For these two techniques, we will discuss the underlying principle and scope as well as the results. Other techniques, such as NMR, infrared, and Raman spectroscopy, have recently been utilized in glycosaminoglycan research; they will be reviewed briefly.

ORD and CD

Although Biot discovered the optical activity of cane sugar over 150 years ago, it is only within the last 20 years that investigators have succeeded in estimating, with a high degree of confidence, the conformation of macromolecules in solution from the measurement of optical rotatory dispersion and circular dichroism The usefulness of these two phenomena as probes of molecular conformation has been amply demonstrated in the analysis of protein and nucleic acid secondary structure, but is less well established in the study of polysaccharides.

ORD and CD are manifestations of the same property of molecules that have asymmetric or dissymmetric groupings of atoms. Both ORD and CD have their origin in the absorption bands of these optically active compounds. They are thus closely related; detailed theoretical formulation of this relationship is given by Moffit and Moscowitz.[178] The theory permits transforming either kind of data to the other (Kronig-Kramer transform). ORD is a dispersive phenomenon and is exhibited at wavelengths far from — as well as near — those characteristic of the electronic transitions that are responsible for the optical activity. On the other hand, CD is an absorptive phenomenon, observable only in the frequency intervals in which absorption occurs. The typical

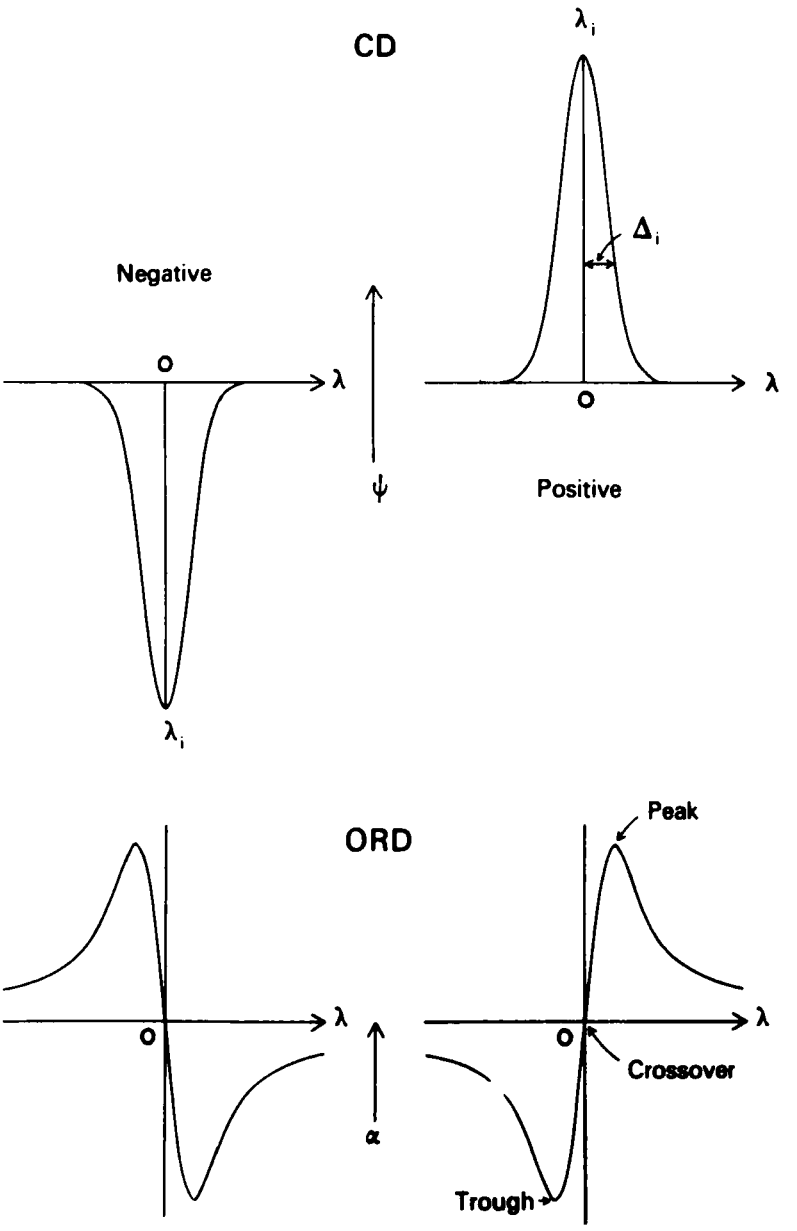


FIGURE 13. Idealized Cotton effect of an isolated optically active absorption band with its maximum at λ_i. Left, negative Cotton effect; right, positive Cotton effect. (From Yang, J. T., in *A Laboratory Manual of Analytical Methods of Protein Chemistry*, Alexander, P. and Lundgren, H. P., Eds., Pergamon Press, New York, 1969, 23. With permission.)

band shapes of ORD and CD for an isolated optically active absorption band are illustrated in Figure 13.

The ORD profiles are commonly called Cotton effects in honor of the French physicist André Cotton, who in 1895 observed the phenomenon in optically active absorbing solutions. The Cotton effect is termed positive if the CD maximum is positive and the peak is on the long-wavelength side, and negative if the CD maximum is negative

and the trough is on the long-wavelength side. In recent terminology, the Cotton effect is referred to the phenomena of both CD and its associated ORD.

Although the electronic absorption bands and the circular dichroism bands are closely related, distinctions must be recognized — it is not a one-to-one relationship. In the absorption spectra, the intensity of absorption depends only on the electric dipole transition moment whereas the intensity of circular dichroism depends on both electric and magnetic dipole transition moments. Thus a very weak absorption band may exhibit intense optical activity, and vice versa. The absorption band may be only weakly allowed or symmetry forbidden, but the associated CD band may be quite strong. Hyaluronate, for instance, exhibits an intense dichroism at 210 nm associated with a very weak $n \rightarrow \pi^*$ absorption band whereas very little optical activity is associated with a strong $\pi \rightarrow \pi^*$ amide transition.

The theory, instrumentation, and method of analysis of CD and ORD have been described by Yang.[179] The methods of measurements and evaluation of these optical parameters are reviewed in a recent article by Stone.[4]

Interpretation of the optical activity of a compound requires an understanding of the optical properties of each of its chromophores. In glycosaminoglycans, the chromophores are the hemiacetal, acetal, hydroxyl, carboxyl, and acetamido groups. The optical characteritics in the observable range of a commercial spectropolarimeter (> 185 nm) are due mostly to carboxyl and acetamido, but the other chromophores also contribute — particularly to the ORD spectrum. Since most of the CD/ORD measurements of glycosaminoglycans have been made with commercial instruments, we will be concerned here primarily with the acetamido and carboxyl chromophores of these polymers.

Glycosaminoglycans have the acetamido substituent in the C-2 position of the amino sugar moiety. Because of the asymmetric environment of the sugar, this amide is perturbed and gives rise to Cotton effects analogous to those of polypeptides. In general, one would expect both the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the amide to show Cotton effects. Beychok and Kabat[180] first proposed that the Cotton effects of *N*-acetylamino sugars in the 200 to 210 nm region might be associated with the $n \rightarrow \pi^*$ amide transition. CD measurements of amino sugars have revealed bands in the 210 nm region that have been assigned to the same transition.[181,182] Kabat et al.[181] have shown that the optical activity may be used empirically to differentiate various intersaccharide linkages and substituent positions. The anomeric configuration at C-1 has been shown to have a strong influence on the optical activity in the 220 nm region. They have made a case for the importance of anomeric configuration in determining the CD of amido sugars at 210 nm. CD data of Coduti et al.[183] do not tend to support this proposal; rather they show that CD in the $\pi \rightarrow \pi^*$ region varies with anomeric configuration, substituent position, and linkage position. The data on model systems[183] substantiate Stone's[184] generalized proposal for CD of glycosaminoglycans that 1 → 4 linked polymers have large positive CD near 190 nm, whereas 1 → 3 linked polymers have small or negative CD in the $\pi \rightarrow \pi^*$ amide transition region.

Also appearing as a chromophore in glycosaminoglycans are carboxyl groups of the uronic acid moiety. Park and Chakrabarti[175] have shown that the C(5) epimer of the uronic acids (glucuronic and iduronic) in glycosaminoglycans can influence significantly the CD properties of the polymer. Iduronic acid is an inherently weaker acid than glucuronic acid,[175,185] and the optical properties of the two are not identical.[186] The galactouronosides, guluronosides, and iduronosides in which O(4) is axial all exhibit a CD band around 210 nm attributable to the $n \rightarrow \pi^*$ carboxyl transition.[186] In contrast, D-glucuronic and D-mannuronic acids and their derivatives give an additional CD band around 235 nm.

Listowski et al.[187] first reported this long-wavelength band and, from solvent-dependent CD studies, assigned it to the n → π* transition of unsolvated molecules and the normal band at 210 nm to the same transition in molecules hydrogen-bonded to water. This interpretation was later rejected, and the most widely accepted explanation of the two bands is that they arise from the n → π* transition of different rotational isomers of carboxyl groups.[186,188] Any rotation of the carboxyl-group chromophore against the dissymmetric environment of the rest of the molecule can cause large changes in CD magnitude, including the position and sign of the band.[186] Studies of the CD bands due to the π → π* amide or carboxyl transition of the monomeric sugars and of their polymer, which are located below 200 nm, were limited experimentally; the range of the commercial spectropolarimeters does not extend below 185 nm and the measurements below 200 nm in many cases include artifacts. With the rapid development in instrumentation over the last few years, it is now possible to measure CD in the vacuum ultraviolet region.[189] We have used this technique successfully and reported the CD of *N*-acetylglucosamine and glucuronic acid in film and in solution (Figures 14—16), extending the measurements down to 170 nm.[190]

The results reveal that *N*-acetylglucosamine exhibits a positive CD band at 188 nm in addition to a negative band near 210 nm. The positive band has been assigned to the π → π* amide transition; alternative assignment to a ring transition is contraindicated by the absence of CD in that region in unsubstituted monosaccharides.[191] The negative band near 210 nm is due to n → π* amide transition. Since unsolvated amides typically have CD bands at higher wavelengths — e.g., 222 nm in α-helical polypeptides — we have concluded that the amide group in *N*-acetylglucosamine in monomer or in polysaccharide is highly solvated.

There is an optically active transition at 182 nm in glucuronic acid and glucuronate, the rotational strength of which is negative in solutions of both species and in films of the acid; in films of the anion its rotational strength is positive.[190] The location of the band is close to what is expected for a carboxyl transition although some contribution from a ring transition cannot be ruled out. The vacuum UV CD of glucose and other monosaccharides[191] exhibits weak ellipticity near 180 nm, and Balcerski et al.[192] and Pysh[193] observed a negative 180 nm band in iota-carrageenan. The 182 nm band may have contributions from both a carboxyl π → π* transition and a ring transition. The change in sign of the CD band in the film relative to the solution was explained[190] by intermolecular as well as carboxyl-cation interactions. In this study, the CD features of glucuronate or glucuronic acid above 190 nm, previously assigned[186,188] to n → π* transition, are attributed to the existence of two rotational isomers, with the negative charge of the anion causing blue shifts relative to the acid (Figures 15 and 16). CD features of the monomeric constituents of glycosaminoglycans provide a basis for the analysis of structure, conformation, and interaction properties of these polysaccharides.

It was not until 1965 that the optical rotatory dispersion down to 188 nm of glycosaminoglycans was reported.[194] They show intrinsic Cotton effects in the regions of their amide and carboxyl transitions, but differences were found in the sign and spectral position of the ORD Cotton effects for heparin (positive) and for chondroitin 6-sulfate (negative). Subsequently, ORD of other glycosaminoglycans were examined.[182,195] In these pioneering studies, Stone showed that all glycosaminoglycans exhibit their first Cotton effect around 208 to 212 nm and that they can be divided into two classes based upon the sign and position of a second Cotton effect below 200 nm. She further pointed out that these polysaccharides show enhanced positive or enhanced negative rotation in comparison to their monomeric units; the rotational strength of the *N*-acetylglucosamine-containing polymer is greater than that of the *N*-

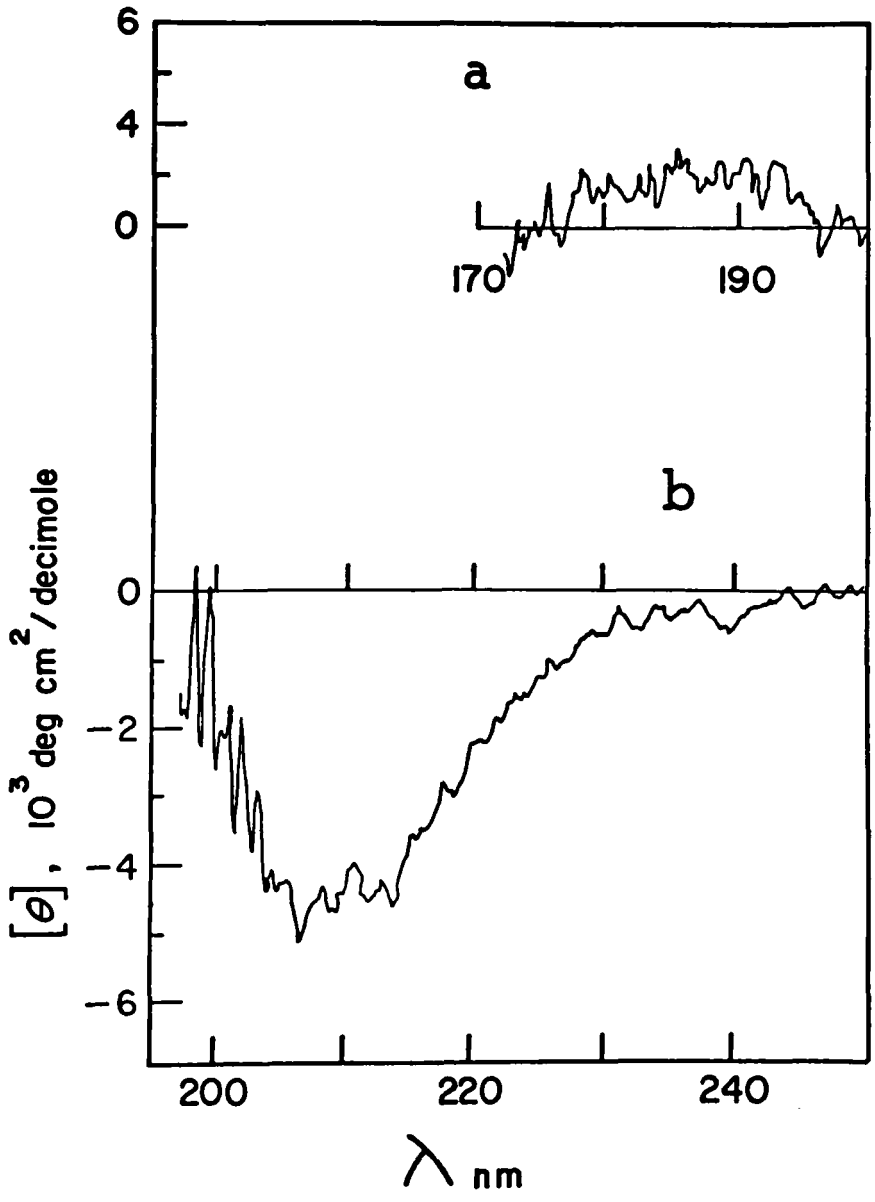


FIGURE 14. Circular dichroism of *N*-acetylglucosamine, 6 mg/m*l*. (a) Pathlength 0.046 mm. (b) Pathlength 0.1 mm. (From Buffington, L. A., Pysh, E. S., Chakrabarti, B., and Balazs, E. A., *J. Am. Chem. Soc.*, 99, 1730, 1977. With permission. Copyright by the American Chemical Society.)

acetylgalactosamine-containing polymer. A degree of preferred order in the polymer molecule was implied from this enhanced rotation.

Ultraviolet circular dichroism studies[184] of glycosaminoglycans gave a better resolution of the two Cotton effects; the first negative CD band centered around 210 nm is common to these polymers, and glycosaminoglycans that contain 4 → 1 linked amino sugars show a second, positive band around 190 nm and those with 3 → 1 linked amino sugars show a second, negative band. Keratan sulfate, heparin, and heparan sulfate belong to the former group; hyaluronic acid, chondroitin sulfate, and dermatan sulfate

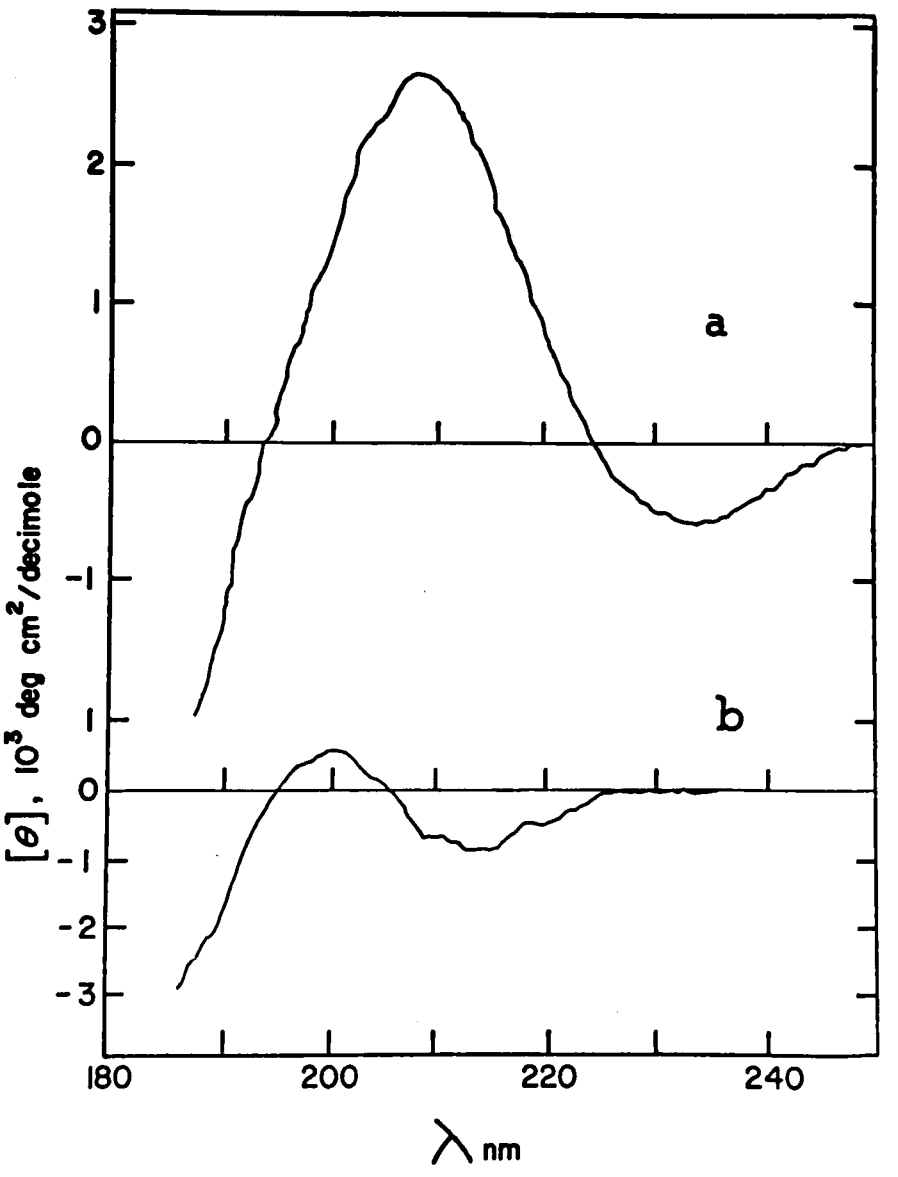


FIGURE 15. Circular dichroism of glucuronic acid, 14 mg/m*l*. (a) At pH 2.5. (b) At pH 6.9 (glucuronate). (From Buffington, L. A., Pysh, E. S., Chakrabarti, B., and Balazs, E. A., *J. Am. Chem. Soc.*, 99, 1730, 1977. With permission. Copyright by the American Chemical Society.)

constitute the latter group. The second CD band has thus been shown to be dependent on the intersaccharide linkage and possibly on the conformational specificity of the polymer.[184] The specific conformational variation for the group of glycosaminoglycans showing a positive CD band below 200 nm has been suggested[3,4] from model building with Courtald space filling atoms — a hydrogen bond between a nitrogen atom and the C-3 (or C-2 in the case of keratan sulfate) hydroxyl group of the following uronic acid residue. For the other groups showing a negative CD band, the hydrogen bond is between the nitrogen atom and the C-2 hydroxyl group of the uronic acid moiety. X-ray diffraction results,[164] however, do not correspond with the proposed model.

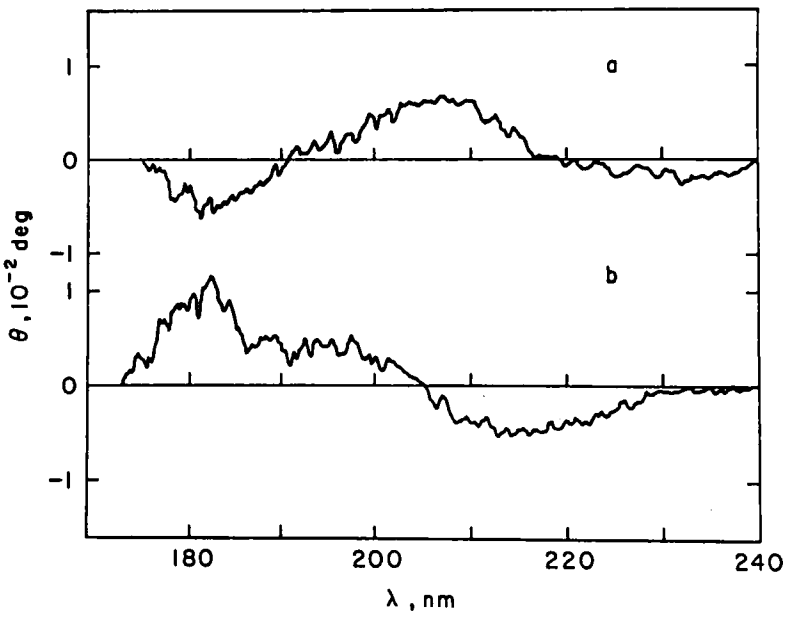


FIGURE 16. Circular dichroism film spectra. (a) Glucuronic acid. (b) Sodium glucuronate. (From Buffington, L. A., Pysh, E. S., Chakrabarti, B., and Balazs, E. A., *J. Am. Chem. Soc.*, 99, 1730, 1977. With permission. Copyright by the American Chemical Society.)

Stone[184] argued that the predominant chromophore in the CD bands of glycosaminoglycans is the acetamido group. The basis for such argument is that keratan sulfate, having no carboxyl chromophore, shows similar CD bands to that of other uronic acid-containing glycosaminoglycans whereas polygalactouronate displays a simple dispersion curve.[3*] The absorption spectra of some glycosaminoglycans and *N*-acetylglucosamine have been reported by Stone[184] (Figure 17).

Hyaluronic acid[345] displays a curve similar in shape and magnitude to that of the chondroitin 4-sulfate absorption spectrum. The $n \rightarrow \pi^*$ amide and carboxyl transitions, located above 200 nm, are obviously weak. The stronger $\pi \rightarrow \pi^*$ amide transitions of uronic acid-containing glycosaminoglycans do not, however, show their absorption maxima, presumably due to the tail absorption of the strong carboxyl transition, located in the far ultraviolet region. This is evident from the fact that both keratan sulfate and *N*-acetylglucosamine display a well-resolved peak around 190 nm. In contrast to the situation with polypeptides, the assignment of CD bands from the absorption spectra is rather difficult because of the fact that both carboxyl and amide chromophores are in the asymmetric environment of the sugar molecule. CD spectra of the monomeric sugars seem to be better suited for this purpose.[190]

Conformation of a polyion has been frequently investigated by observing the pH-dependent optical properties of such molecules. Heparin was the first molecule to be studied in detail.[196] ORD studies[195] of heparin revealed a first negative Cotton effect with a trough near 230 nm. The increased negativity of this Cotton effect around 230 nm at lower pH values was attributed to a conformational change of the molecule (Figure 18).

* Grant and co-workers[3*] reported a CD peak of polygalactouronate around 202 nm, and J. N. Liang and E. S. Stevens[346] obtained, in addition to the positive CD, a negative band below 200 nm of the same magnitude.

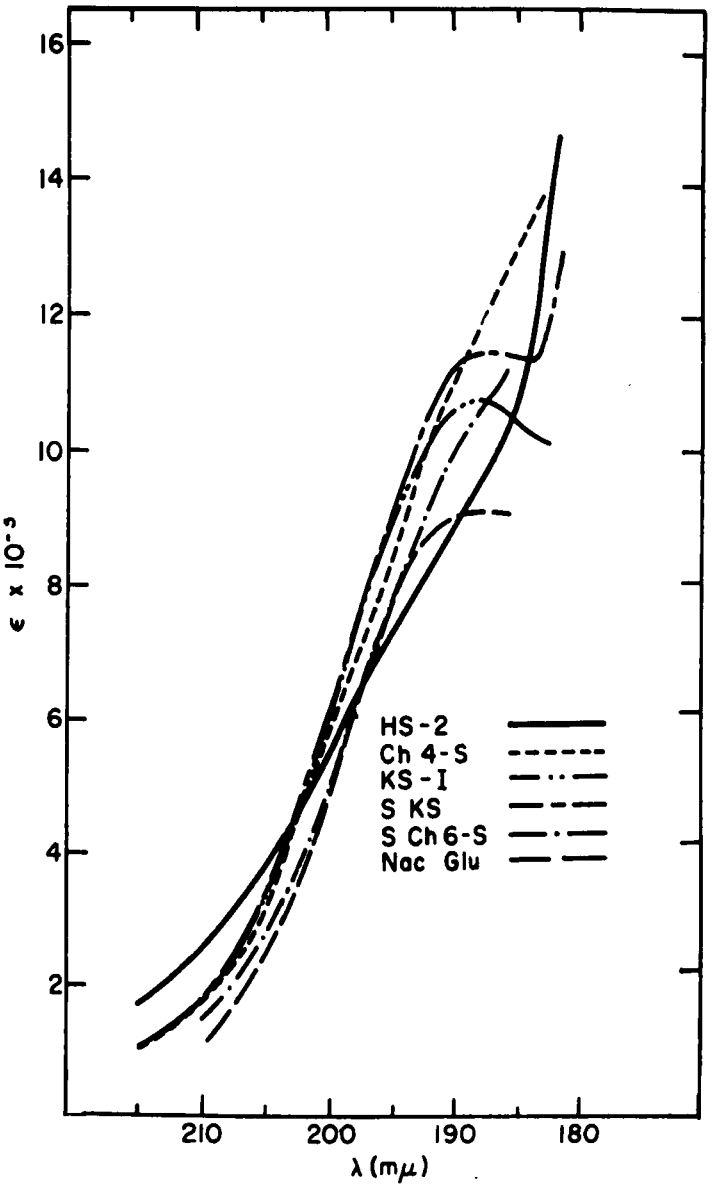


FIGURE 17. Absorption spectra (215-183 mμ) for glycosaminoglycans. Measurements were made in the Cary Model 15 spectrophotometer under nitrogen purge at room temperature. Solutions were 0.5-1.2 mg/mℓ in distilled water. The optical pathlength was 0.5 or 1.0 mm. Extinction coefficients were computed on the basis of the average formula weight of *N*-acetylglucosamine moiety. S KS and S Ch 6-S are oversulfated keratan sulfate and chondroitin 6-sulfate, respectively. Nac Glu is *N*-acetylglucosamine. (From Stone, A. L., *Biopolymers*, 10, 739, 1971. With permission.)

The formation of a histamine-heparin complex was shown to cause an analogous change in the ORD of heparin with no change in pH.[195] Similar results were obtained[196] in the CD studies of heparin; a twofold increase in the negative CD band at 210 nm occurs when the pH is lowered from neutral to 2.4. The significance of these changes as a reflection of conformational alteration in heparin is obscured by the fact that the

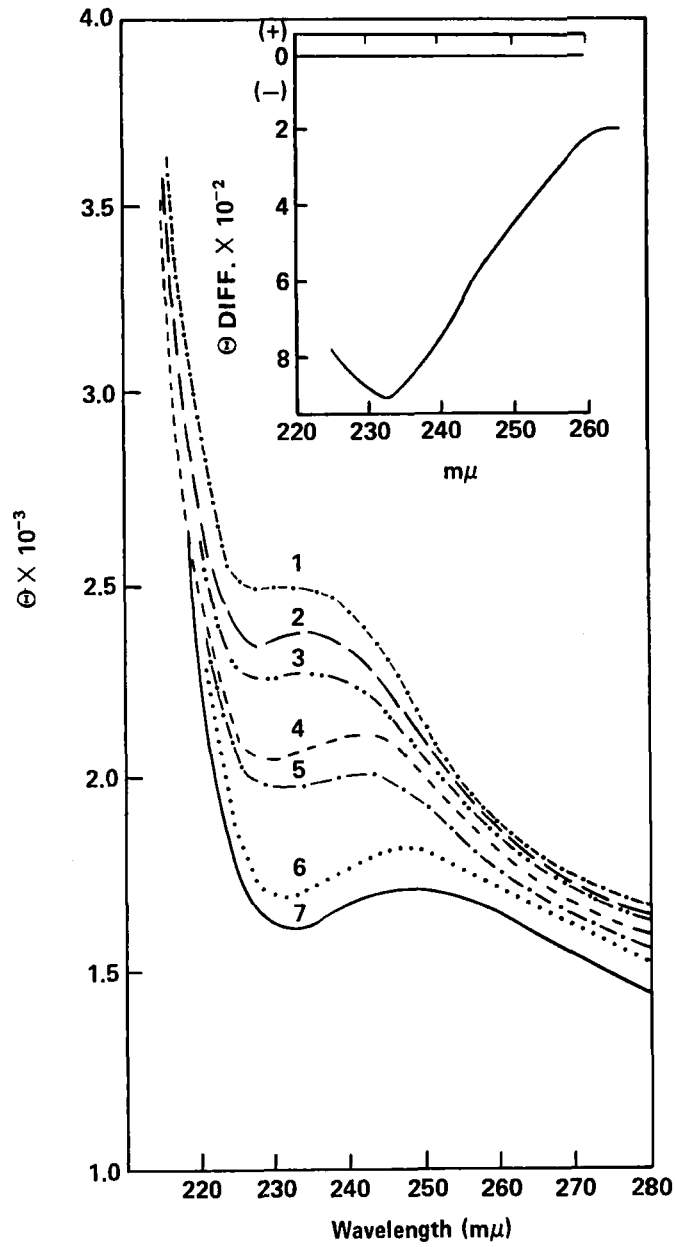


FIGURE 18. Changes with pH of heparin ORD in the spectral range 220-280 mμ. Curves 1-7 are those at pH 7, 6.1, 5.5, 5.0, 4.4, 3.1, and 2.7, respectively, for a given heparin solution, 1.52 mg/mℓ, at a pathlength of 0.5 cm. Inset is the difference curve between curves 1 and 7. ϕ values are uncorrected for the refractive index of the water solvent. (From Stone, A. L., *Nature (London)*, 216, 551, 1967. With permission.)

carboxyl group may make some contribution to the observed pH-dependent optical properties.

Detailed circular dichroism studies of glycosaminoglycans including chemically transformed heparins at various pH values have indeed revealed that the carboxyl

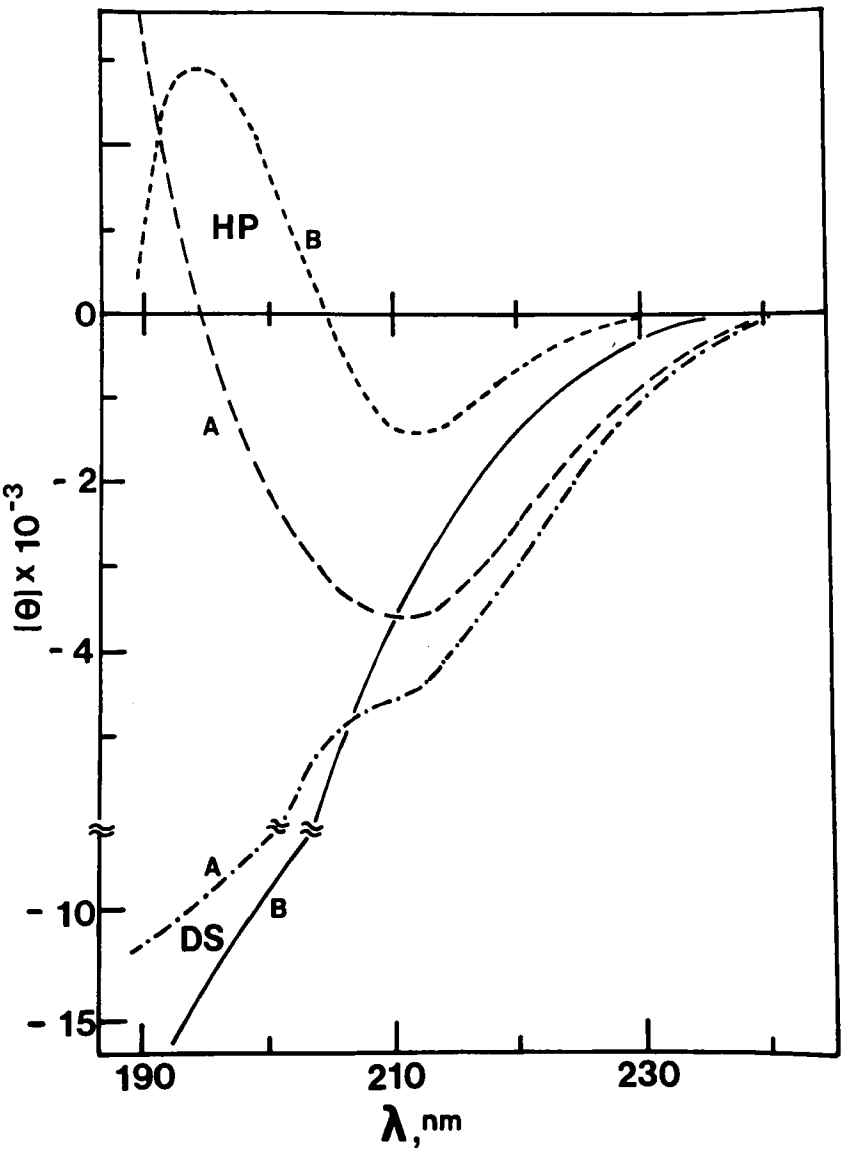


FIGURE 19. Circular dichroism of dermatan sulfate (DS) and heparin-2 (HP). (A) pH 2.6. (B) pH 7.5. (From Park, J. W. and Chakrabarti, B., *Biochim. Biophys. Acta*, in press. With permission.)

chromophore plays an important role in the dichroic behavior of these polymers.[175,197] We have demonstrated that variations of CD spectra of heparin above 200 nm with pH arise mainly from the acid-base behavior of the uronic acid moieties, and the phenomenon can be successfully utilized to determine the apparent pK_a value of the polymer.[197]

The pH-dependent CD features of glycosaminoglycans above 200 nm can be distinguished by their uronic acid moieties; with decreasing pH, iduronic acid-containing polymers show increased negative ellipticity near 220 nm whereas those containing glucuronic acid display enhanced negative dichroism near 230 nm and decreased negative dichroism near 210 nm.[175] For predominantly iduronic acid-containing samples,

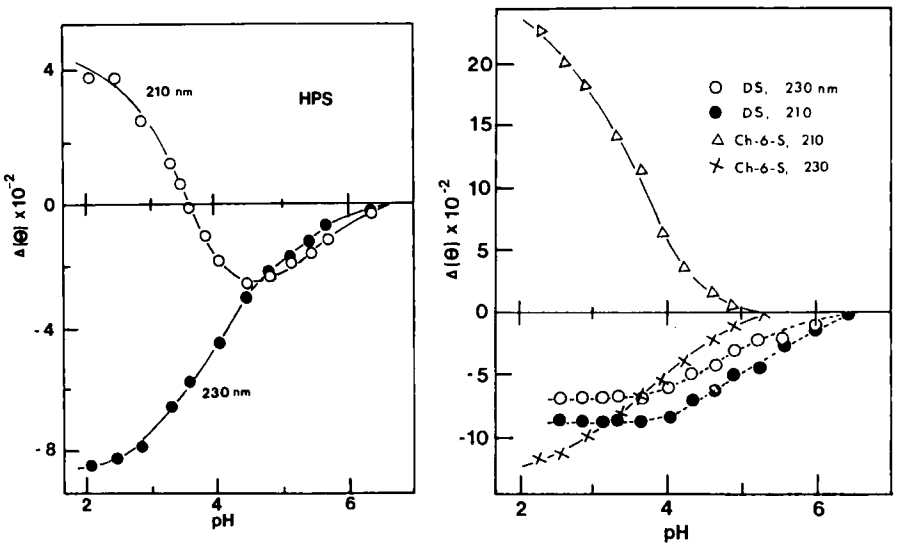


FIGURE 20. Ellipticities at various pH's. (A) DS, dermatan sulfate. Ch-6-S, chondroitin 6-sulfate. (B) HPS, heparan sulfate. (From Park, J. W. and Chakrabarti, B., *Biochim. Biophys. Acta*, 544, 667, 1978. With permission.)

heparin and dermatan sulfate, the ellipticity values at both 210 and 230 nm became more negative with decreasing pH (Figures 19 and 20). The difference in the acid-induced dichroic behavior of different uronic acid (C-5 epimers)-containing polymers is evident (Figure 20). Heparan sulfate, which contains both uronic acids, shows the combined effects of both glucuronic acid and iduronic acid.

If the change in CD features at a particular wavelength that occurs with change in pH is due solely to one chromophore, and no major conformational transition is associated with the change, then the relative variation of ellipticity is expected to follow an empirical equation:[198]

$$\mathrm{pH} = \mathrm{p}K_a - n \log \left(\frac{\alpha}{1-\alpha}\right) \qquad (8)$$

where α denotes the degree of dissociation and can be related to ellipticity change as $\alpha = (\theta - \theta_{pH} \ll {}_{pKa})/(\theta_{pH} \gg {}_{pKa} - \theta_{pH} \ll {}_{pKa})$. An empirical constant (n) depends on the ionic strength and the concentration of the solution. The apparent dissociation constant of the chromophore ($\mathrm{p}K_a$) can thus be determined from Equation 8.

With this rule of thumb, it has been possible for us to examine whether the pH variation can cause any conformational change of the molecule — the changes in ellipticity at 230 nm for hyaluronic acid, chondroitin, and chondroitin sulfate follow Equation 8, but the changes at 210 nm do not. The reverse is true for heparin, *N*-desulfated heparin, and *N*-acetylated heparin. The fact that the 230 nm ellipticity values of the first group of polymers follow Equation 8 indicates that the change in CD properties in this wavelength region is due entirely to the acid-base properties of the carboxyl group. Protonation of the acetamido group and/or conformational variation[111] may contribute to the dichroic behavior at 210 nm, and thus the ellipticity changes at this wavelength do not obey the equation. Except for dermatan sulfate, compounds of the second group contain both iduronic acid and glucuronic acid (Table 1). Thus the ellipticity variations with pH at both 210 and 230 nm can be used for dermatan sulfate to

determine the pK value. Even though heparin contains a small amount of glucuronic acid, the change in dichroism at 210 nm follows Equation 8 because the variation with pH at this wavelength for iduronic acid-containing polymer is much larger than that for glucuronic acid-containing polymer. Because of the presence of a considerable amount of both glucuronic and iduronic acids, the method is not applicable to heparan sulfate.

The lower ellipticity values of dermatan sulfate, heparin, and heparan sulfate near 210 nm compared to their monomeric constituents[175,184] and other glycosaminoglycans suggest that the $n \rightarrow \pi^*$ transition of amide groups is weakly optically active or virtually inactive.* Even *N*-acetylation of heparin does not increase the ellipticity values at 210 nm. Park and Chakrabarti[175] proposed a general rule that a disaccharide unit containing *N*-acetylamino sugar linked with iduronic acid would display either a weak $n \rightarrow \pi^*$ amide CD band or none. This is supported by the fact that heparan sulfate shows a larger dichroism than does *N*-acetylated heparin at 210 nm, because of the former's relatively higher content of both glucuronic acid and *N*-acetylglucosamine.

X-ray studies[165] favor the 1-C conformation of iduronic acid in heparin as opposed to the C-1 in dermatan sulfate, but NMR studies in solution[177] do not suggest such a difference. The conformation of the L-iduronic acid in dermatan sulfate has been suggested as C-1, based on analysis of optical rotatory dispersion curves.[176] The striking dissimilarities in the dichroic properties of heparin and dermatan sulfate in their acid and salt forms (Figure 21) and in the CD spectra of the copper complexes (Figure 22) have been attributed either to their difference in intersaccharide linkage or to the conformation of the iduronic acid moiety.

The spectral behavior of heparin in the $\pi \rightarrow \pi^*$ transition region associated with change in pH might originate from conformational change, as has been suggested for hyaluronic acid.[111] However, the CD features above 200 nm provide no evidence[98,175] for any major conformational transition of glycosaminoglycans with change in pH, but the configurational differences of the uronic acid moiety[174,175] are well reflected in the dichroic behavior of the polymer, and the carboxyl chromophore thus plays a major role in the chiroptical properties of the molecules.

From the anomalous dispersion in the absorption band of the bound dye, Stone[194] suggested a card-stack aggregation of dye molecules with a helical conformation for the heparin-dye complex. (A detailed discussion of the phenomenon is included later in this article.) She demonstrated further that the induced Cotton effects diminished with graded hydrolysis of the polymer. The phenomenon of induced rotation was investigated by Stone and Moss,[199] as a function of pH, in the range of ionization of the carboxyl group of heparin; they found that metachromacy decreased with a decrease in anionic site density. Further investigations by Stone[200] on dye binding indicated that the binding of methylene blue to the anionic sites of heparin gives rise to an exciton-like CD doublet. The positive sign of the low-frequency band for the heparin-methylene blue complex of the doublet has been attributed to net left-handed chirality for the neighboring dye molecules. Changes in conformation of heparin were also followed through ORD by Hirano and Onodera.[201] Their study indicates that *N*-desulfation does not change the original conformation, but complete desulfation causes the helical conformation of the heparin-dye complex to disappear.

* Park and Chakrabarti[175] recently reported that dermatan sulfate near pH 7.0 exhibited neither a CD minimum near 210 nm nor an ORD trough above 200 nm. This finding differs from a report of a weak CD band at the wavelength[184] and from ORD results of Davidson,[176] but is in good agreement with the data of Suzuki et al.[42] It is likely that the samples of dermatan sulfate used by Stone[184] and Davidson[176] had some glycosaminoglycan impurities.

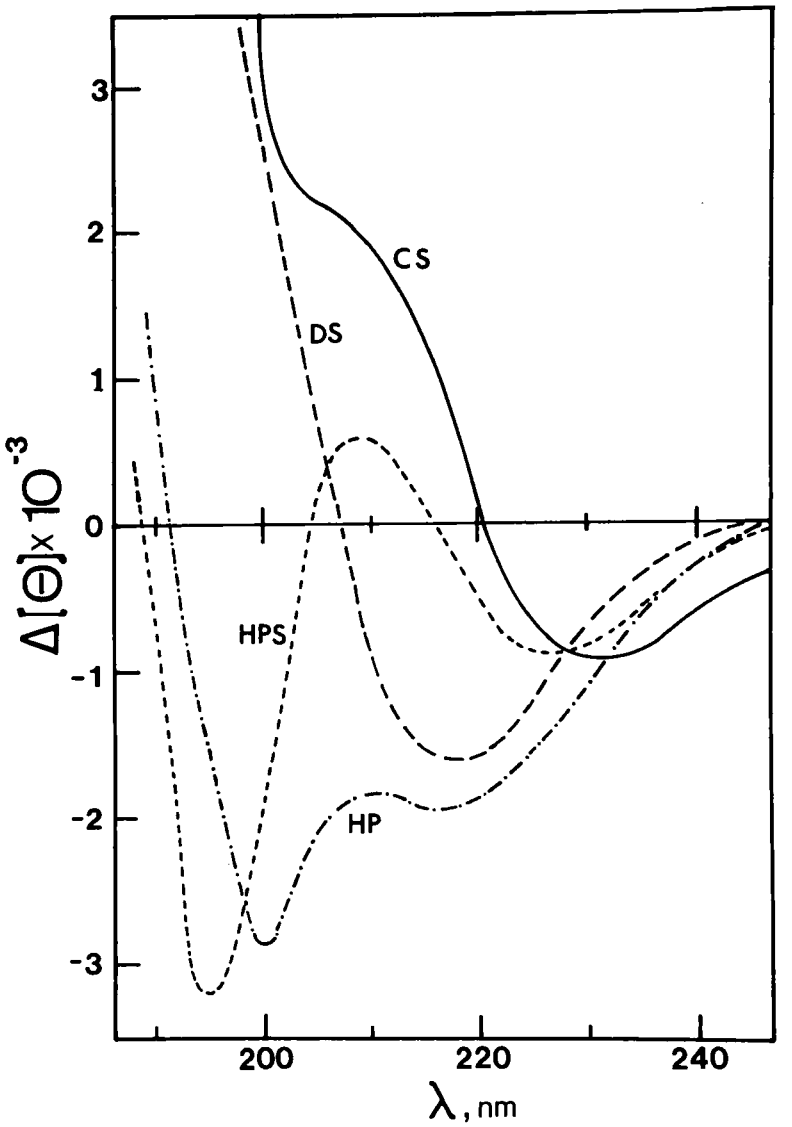


FIGURE 21. Difference CD spectra $\Delta\theta$ ($\theta_{pH2.6}$-$\theta_{pH7.5}$), of chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HPS), and heparin (HP). The behaviors of hyaluronic acid and *N*-desulfated heparin are similar, in shape and wavelength, to those of chondroitin sulfate and heparin, respectively. (From Park, J. W. and Chakrabarti, B., *Biochim. Biophys. Acta*, 544, 667, 1978. With permission.)

Chung and Ellerton[106] suggested a conformational change of heparin when Cu(II) binds to the molecule. The interpretation was based on the observation that the CD band of heparin at 210 nm shifts to 235 nm after binding of the metal ion. Mukherjee et al.,[174] however, attributed the phenomenon to the change in the spatial orientation of the carboxyl group of the polymer rather than to a major macromolecular conformational transition. The chelation of carboxyl and sulfamino to the metal ion facilitates the change in the spatial orientation of these groups (or vice versa).

The chiroptical properties of hyaluronic acid have been studied extensively by Chakrabarti and co-workers.[91,111-115,190,202-204] Chakrabarti and Balazs[111] reported that the molar optical rotation at 220 nm and ellipticity values at 210 nm are greatly enhanced

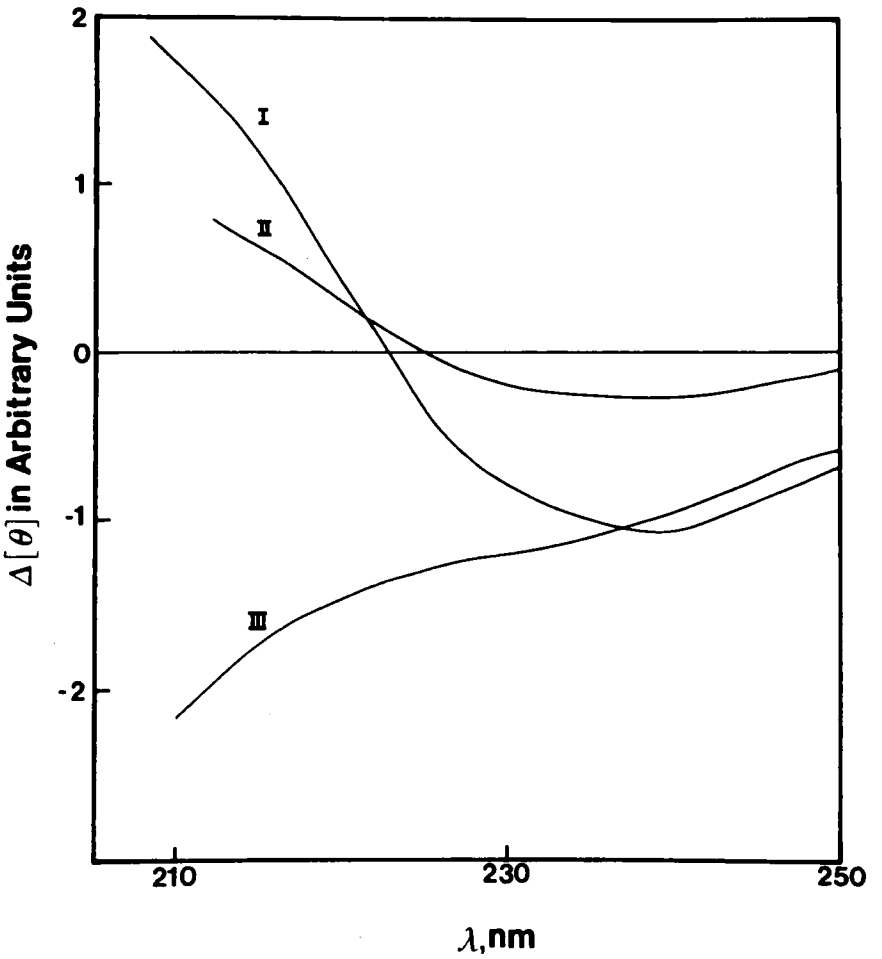


FIGURE 22. Difference CD spectra, $\Delta\theta$ ($\theta_{polymer\text{-}Cu^{2+}} - \theta_{polymer}$), of (I) heparin-$Cu^{2+}$, (II) heparan sulfate-$Cu^{2+}$, and (III) dermatan sulfate-Cu^{2+} at pH 5.9. (From Mukherjee, D. C., Park, J. W., and Chakrabarti, B., *Arch. Biochem. Biophys.*, 191, 393, 1978. With permission.)

in comparison to the values for the monomeric units and oligosaccharides. The second negative CD band below 200 nm was found to change abruptly to the positive side when the pH of the solution is below 4, and it reaches a maximum value at pH 2.5. This positive CD band has been shown to be temperature and concentration dependent. The results were attributed to the conformational change of a single polysaccharide chain or to a chain-chain interaction. Further analysis[205] of CD and ORD data of hyaluronic acid at low pH indicated that the high magnitude of the CD band at 188 nm might be due to the development of internal structure rather than a conformational change.

Hirano and Kondo-Ikeda[206] suggested an ordered conformation of hyaluronate which changes to random coil after treatment with urea. The suggestion was based on the observed changes in the elution pattern and optical rotation at sodium-D light. The data on the extrinsic Cotton effect of the hyaluronate-acridine orange complex support this view and also indicate the left-handed chirality of the hyaluronate helix.[202] A change in the chiroptical properties of hyaluronate at pH near 12 has been attributed to order-disorder transition of the polymer.[203] A similar suggestion was made by Ma-

thews and Decker[92] from their viscosity measurements of hyaluronate at high pH. For hyaluronate solutions at 0.1 ionic strength and pH 7 to 7.5, Barrett and Harrington[93] described changes in intrinsic viscosity and in the rotational diffusion constant calculated from flow birefringence. The phenomena may reflect a change in intermolecular network structure rather than a molecular conformational change.

Vacuum UV CD study[190] has provided further detailed analysis of the phenomena. Several possibilities for the nonadditivity of the CD band at 210 nm of hyaluronate were considered: the change in the configuration of the sugar ring and/or its hydroxyl groups in the presence of neighboring sugar rings, contributions of polarizability and static field to the optical activity of the $n \rightarrow \pi^*$ transition of the substituent groups, or the alteration of the orientation of the acetamido or carboxyl group due to neighboring residues. The study, however, has not ruled out the possibility of an ordered structure that could be responsible for the nonadditivity of the CD intensity near 210 nm. The large rotational strengths of the $n \rightarrow \pi^*$ amide transition in solution and $\pi \rightarrow \pi^*$ transition in film of hyaluronate in comparison to those of chondroitin 4-sulfate have also been attributed to some conformational constraints in hyaluronate.[207]

The positive CD band near 188 nm observed in hyaluronic acid[190] has been confirmed by vacuum ultraviolet CD and assigned to the $\pi \rightarrow \pi^*$ transition of the amide group. This study emphasized that CD is not a good probe for the investigation of conformational change of the molecule at lower pH although the change in intensities of both 188 nm and 210 nm bands cannot always be explained[175] in terms of protonation of the carboxyl groups. However, vacuum ultraviolet study has clearly indicated that the positive band at 188 nm is not due to a ring transition.[205]

Of major interest is the CD spectrum of hyaluronate film (see Figure 27), which was prepared from the same type of hyaluronate solution and approximately in the same way as the film studied by X-ray diffraction.[159] The film shows a large band near 194 nm which we have assigned to the $\pi \rightarrow \pi^*$ amide band, and $n \rightarrow \pi^*$ bands are hidden in the long-wavelength tail of the band. The large rotational strength of the CD band has been attributed to a decreased rotational freedom of the acetamido group when it participates in an intramolecular hydrogen bond with the carboxyl group of the neighboring residue. Buffington et al.[190] suggested that the conformation of hyaluronate in film may be a fourfold helical structure similar to that reported from X-ray diffraction studies.[159] This is supported by the fact that chondroitin 4-sulfate film, in which there is no possibility for a hydrogen bond between the acetamido C-4 and glucuronate ring oxygen, exhibits significantly lower ellipticity (about 1×10^3 deg · dm²/decimole) than that of hyaluronate.[207]

Considerable progress has been made in the studies of conformation of hyaluronic acid in solution since Chakrabarti[190] first observed a dramatic change in the chiroptical properties of the molecule in ethanol-water solvent. Later, Park and Chakrabarti[112,113] ascribed the changes in CD, ORD, and viscosity of the molecule in organic-water solvents such as ethanol, *p*-dioxane, and acetonitrile/water at $pH \leq pK_a$ to a major conformational transition of the molecule. Under these conditions hyaluronic acid exhibits a strong negative dichroism (below 200 nm) and a positive CD near 226 nm (Figure 23). It undergoes a sharp, cooperative transition with respect to pH (Figure 24) and solvent.

Neither of the monomers (*N*-acetylglucosamine or glucuronic acid) show any significant change in their chiroptical properties with varying solvent and pH. It has been suggested[112,113] that the strong negative dichroism of hyaluronic acid below 200 nm in mixed solvent arises mainly from the $\pi \rightarrow \pi^*$ amide transition. In that case, the large rotational strength of the band indicates a decreased mobility of the acetamido group, possibly due to hydrogen bonding as observed in hyaluronate film.[190]

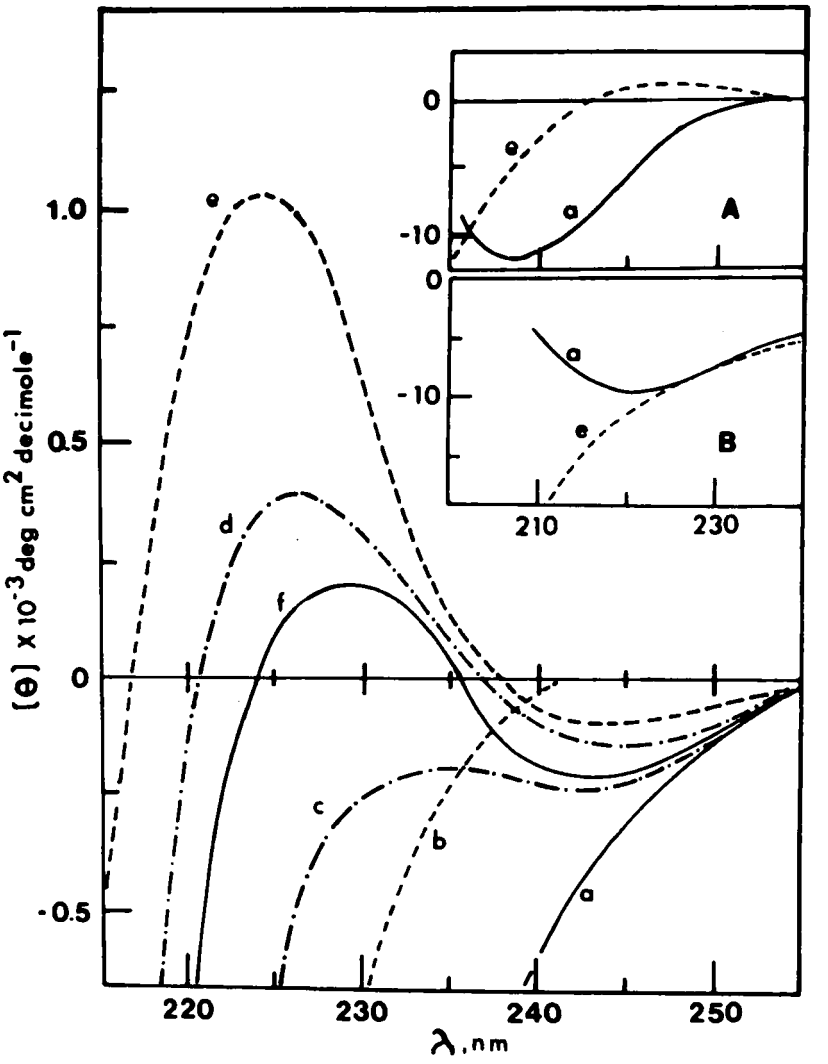


FIGURE 23. Typical variations of CD spectra upon changing ethanol and pH of hyaluronic acid solutions: (a) 0% ethanol, pH 2.6; (b) 0-50% ethanol, pH 6.5; (c) 5% ethanol, pH 2.6; (d) 10% ethanol, pH 2.6; (e) 20% ethanol, pH 2.6; (f) 20% ethanol, pH 3.3. Insets are spectra showing changes in (A) CD peak and (B) ORD trough. The scale of ORD is same as that of CD. (From Park, J. W. and Chakrabarti, B., *Biopolymers*, 16, 2807, 1977. With permission.)

The positive band at 212 nm in the difference spectrum (appearing as a positive band at 226 nm in the CD spectrum) may result from both the 210 nm $n \rightarrow \pi^*$ carboxyl and $n \rightarrow \pi^*$ amide transitions. The magnitude of this band should reflect the algebraic summation of the rotational strengths of all transitions in that region.

Similarly, ORD results[113] showed a gradual blue shift of the trough at 220 nm and enhanced magnitude of rotation with increasing concentration of organic solvents and/or decreasing pH. The shift can be explained on the basis of the resultant effect of two CD bands. The most frequent representation of rotatory dispersion is given by Drude as the summation of individual transition i in the wavelength λ_i;

$$[\alpha] = \Sigma_i \frac{A_i \lambda_i^2}{\lambda^2 - \lambda_i^2} \qquad (9)$$

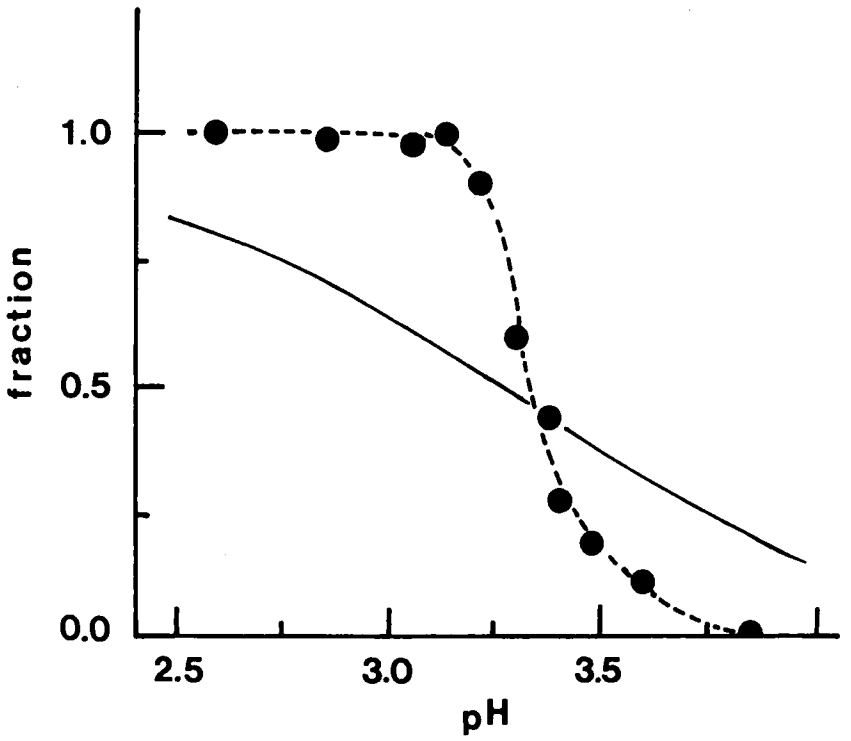


FIGURE 24. Relative changes of difference CD spectra of hyaluronic acid (about 0.5 mg/ml) at 212.5 nm vs. pH. Solid line is calculated fraction of ideal undissociated monobasic acid of pK_a = 3.23. (From Park, J. W. and Chakrabarti, B., *Biopolymers*, 17, 1323, 1978. With permission.)

Beychok and Kabat[180] studied the ORD of the maltose and amylose series of oligosaccharides using a one-term equation, Equation 9, and showed that the λ_c is about 150 nm, whereas hyaluronic acid is expected to have a λ_c value between 180 and 210 nm due to its chromophore transitions. Near a particular transition, the ORD data reflect mainly that transition. However, far from any transition, the values of rotation are determined by all transitions with a magnitude roughly proportional to $A_i\lambda_i^2$. The increased rotation above 300 nm could be due to the higher contribution from the sugar ring. The variations of λ_c and A_c values with solvent composition parallel those of $f\Delta_{(CD)}$, qualitatively, and are shown in Table 4. Since $f\ \Delta_{(CD)}$ values are roughly proportional to the fractions of the new conformational state, which exhibits strong negative dichroism and optical rotation below 200 nm, the increase in $f\ \Delta_{(CD)}$ consistently follows the decrease in λ_c and increase in A_c values. The λ_c values, as low as 182 nm, account well for the absence of CD minima above 187 nm in the new conformational state.

The overall changes in CD and ORD and the lower values of intrinsic viscosity of hyaluronic acid in mixed solvents have been attributed to conformational transition of the moleculae. All the observed property changes of hyaluronic acid are reversed on addition of formamide, indicating that the hydrogen bondings are involved in this transition.

The CD results with different solvents in our study did not show any direct relationship with their dielectric constants. The results have been explained in terms of charge distribution and change in the solvation properties in the domain of the polymer chain. The properties of aqueous hyaluronic acid solution at pH about 2.5 were explained in

TABLE 4

ORD Parameters and Relative CD Changes of Hyaluronic Acid[a]

Solvent[b]		ORD		CD
pH	Ethanol (%)	λ_c (nm)	A_c (10^3 deg·cm²dmole^{-1})	$[f\Delta_{(CD)}]^c$
2.6	0.0	204	1.9	0.00
2.6	2.5	197	2.6	0.16
2.6	5.0	195	2.9	0.58
2.6	7.5	187	3.7	0.79
2.6	10.0	186	3.8	0.89
2.6	10.0[d]	210	0.8	−0.60[e]
2.6	20.0	182	3.9	1.00
3.3	20.0	184	4.1	0.60
3.4	20.0	195	3.2	0.43
6.7	20.0	210	1.5	−0.10[e]

[a] Concentration of hyaluronic acid is ~0.5 mg/mℓ.
[b] All solvents contain 0.1 *M* NaCl.
[c] $f_{\Delta(CD)}$ is calculated from difference CD spectra at 212.5 nm and normalized to 1 for 20% ethanol, pH 2.6.
[d] Contains 1% formamide.
[e] At these conditions, molar ellipticity is more negative than that of aqueous solution, pH 2.6.

(From Park, J. W. and Chakrabarti, B., *Biopolymers*, 17, 1323, 1978. With permission.)

terms of charge balance between negative on carboxyl and positive on acetamido groups.[109] The addition of less polar solvent could disrupt this balance and promote protonation of carboxylate while suppressing the protonation of the amido groups. It is also known that the side groups of hyaluronic acid are highly solvated.[190] The water molecule forms a cluster around the charged sites of the polymer.[208] At a critical point in the solvent concentration and in the charge distribution between the amido and carboxyl groups, a cooperative depletion of water molecules from the clusters favors the transition to a new conformation.

To explore the nature of the transition and the thermal stability of the conformation, Park and Chakrabarti[114] extended these studies at various temperatures using hyaluronic acid, methyl hyaluronate, and chondroitin. Neither methyl hyaluronate nor chondroitin undergoes conformational transition in organic-water solvent, as is observed for hyaluronic acid. The CD change of low-molecular-weight hyaluronic acid is considerably smaller than that of the high-molecular-weight acid, indicating that the stability of the new conformation is dependent on the molecular weight of the polymer.

The CD property of methyl hyaluronate in acidic ethanol/water suggests that the carboxyl group is directly involved in the conformational transition of hyaluronic acid. The difference in behavior of the titration curves (Figure 25) in the presence or absence of the conformational transition provides conclusive evidence of direct involvement of the protonated carboxyl group.

The variation of pH during titrations of hyaluronate in aqueous solution and of glucuronic acid can be explained by the simple acid-base equilibria of the molecules. Cooperative conformational transition of hyaluronic acid promotes protonation of the carboxyl group. Since H^+ for the protonation is provided by the solution media, the pH of the solution decreases (Figure 25A) or increases (Figure 25B) with conformational change.

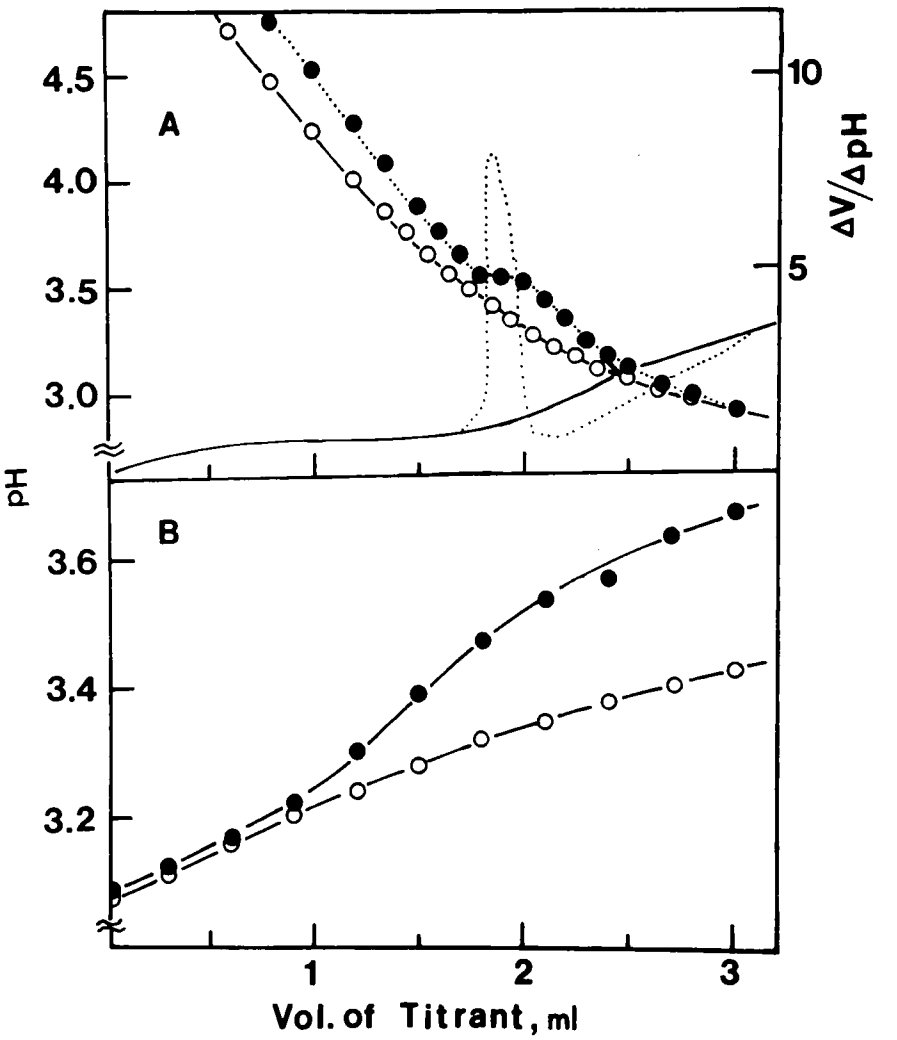


FIGURE 25. Titrations of hyaluronic acid inducing conformational transition. (A) 2 m*l* of 2 mg/m*l* hyaluronate with $6 \cdot 10^{-3}$ *M* HCl aqueous solution (o) and in 20% ethanol (•) Curves without circles are differentials of respective titration curves. (B) (•) 2 m*l* of 2 mg/m*l* hyaluronic acid, and (o) 2 m*l* of 1 mg/m*l* glucuronic acid in aqueous solutions are titrated with 50% ethanol, pH 5.51. (From Park, J. W. and Chakrabarti, B., *Biochim. Biophys. Acta*, 541, 263, 1978. With permission.)

The temperature dependence of the changes in ellipticity at 225 nm, optical rotation at 220 nm, and viscosity values of hyaluronic acid solution in 10% ethanol, pH 2.6, were studied; the results are shown in Figure 26. Sharp and cooperative transition is evident. The thermal transition is reversible, but the process is rather slow — the original conformation is achieved by leaving the solution at about 4°C overnight.

Thus, the intramolecular hydrogen bonding between carboxyl and acetamido groups as allowed in the fourfold helical structure[159] has been proposed for the conformation of hyaluronic acid in an acidic ethanol/water solvent; the conformation is dependent on pH, solvent, molecular weight, and temperature. The direct participation of the protonated carboxyl group in the new conformation suggests that the hydrogen bonding is between carboxyl hydrogen and the carbonyl oxygen of the acetamido group, rather than between the carboxyl and the amide hydrogen suggested in hyaluronate

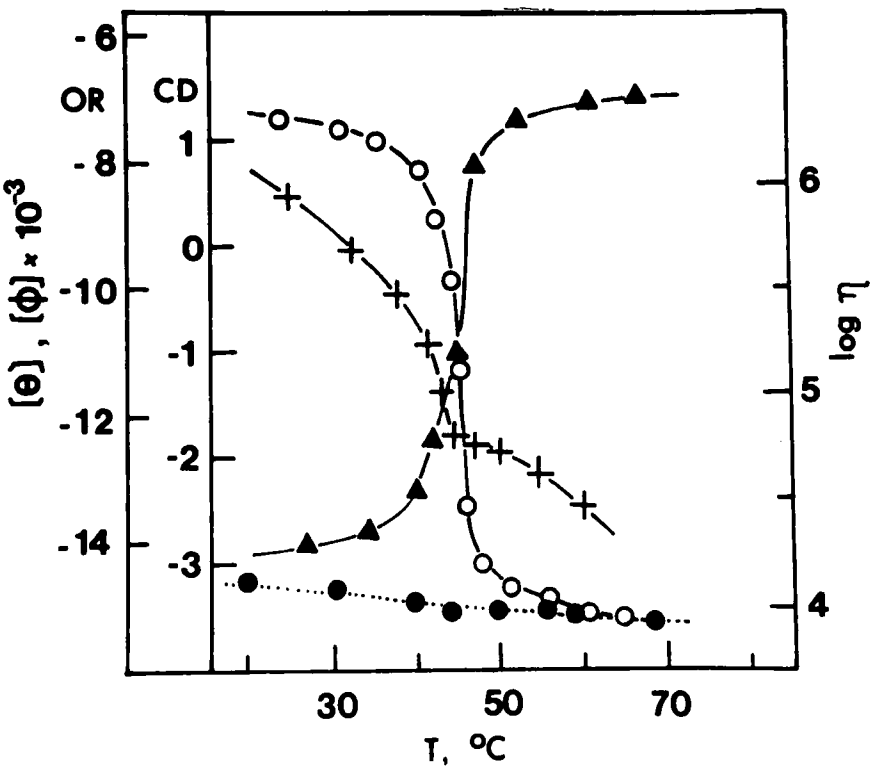


FIGURE 26. Plots of CD at 225 nm (o), optical rotation at 220 nm (▲), and ln η (x) against temperature of 0.4 mg/m*l* hyaluronic acid solution in 10% ethanol, pH 2.6. Solid circles indicate CD ellipticity at 225 nm obtained from aqueous solution, pH 2.6. Ellipticity values are expressed in deg·cm²/dmol; viscosity values are relative quantities. (From Park, J. W. and Chakrabarti, B., *Biochim. Biophys. Acta*, 541, 263, 1978. With permission.)

film.[190] In both cases, $\pi \rightarrow \pi^*$ amide transitions were observed to be highly optically active in the CD spectra. Since the intramolecular conformational transition is accompanied by a change in the orientation of the sugar ring with respect to the glycosidic linkage, and the energy that stabilizes the new conformation is so weak, the conformational stability can be achieved only by cooperative transition. This process is likely to be all or none. The gradual thermal transition observed, especially in low ethanol concentration or higher pH, could be due to the polydispersity of hyaluronic acid rather than the equilibrium between two configurations within a single molecule, which is sterically highly unfavorable.

A similar fourfold structure has been proposed by Figueroa and Chakrabarti[115] for Cu^{2+}-hyaluronate at pH values between 6 and 8. Upon addition of Cu^{2+} to hyaluronate solution, three different types of complexes — depending upon pH and concentration of the polymer — can be identified. The first (complex I), which is formed between pH 3 and 6, involves mainly the carboxyl groups of the polymer as ligands and is characterized by a strong absorption band at 238 nm.[209] The second (complex II) forms between pH 6 and 8 and shows a major change in the CD properties. The changes include (1) a new positive CD band at 250 nm and a strong negative one in the $\pi \rightarrow \pi^*$ amide transition region and (2) the disappearance of the negative band in the $n \rightarrow \pi^*$ amide CD band near 210 nm (Figure 27). In complex II, the involvement of nitrogen of the deprotonated acetamido group (in addition to the carboxyl group) in chelation to Cu^{2+} has been suggested. A sharp increase in absorbance at 238 nm from complex I

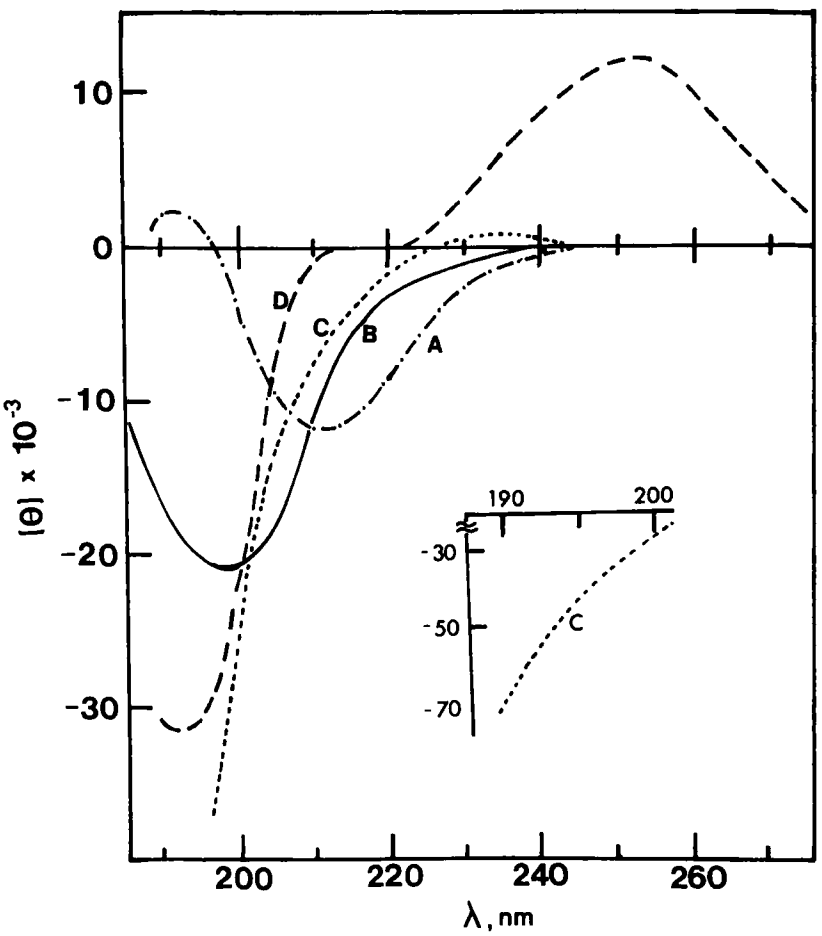


FIGURE 27. CD spectra of hyaluronic acid at pH 3.0 (A), hyaluronate film (B), hyaluronic acid in 20% ethanol at pH 3.0 (C), and Cu^{2+}-hyaluronate complex at pH 6.6 (D). Hyaluronate at pH 7.0 exhibits the same spectra except for slight increase in ellipticity near 210 nm and absence of the positive band near 190 nm. (From Chakrabarti, B., Figueroa, N., and Park, J. W., in *Proceedings of the 4th International Symposium on Glycoconjugates*, Vol. 1, Academic Press, New York, 1979, 119. With permission.)

to II has been attributed to conformational transition which is also manifested in the CD features of hyaluronate. At higher concentration of the polymer or at higher pH, complex II aggregates to a gel, complex III. Chondroitin, in this case also, does not show a CD change in the presence of Cu^{2+}.

A generalized concept[210] regarding the structure of hyaluronic acid in solution has been formulated. Figure 27 shows how the CD spectra of hyaluronate film, hyaluronic acid in aqueous-organic solvent, and Cu^{2+}-hyaluronate complex (pH 6.6) differ from those of acid and neutral forms of hyaluronate.

In all three spectra the appearance of a strong negative band in the $\pi \rightarrow \pi^*$ amide transition region and the apparent loss of $n \rightarrow \pi^*$ CD minima are evident. It has been proposed that, in film and in aqueous-organic solvent, the hydrogen bonding between the carboxyl and acetamido groups restricts the mobility of the latter, giving rise to a strong dichroism in the $\pi \rightarrow \pi^*$ amide transition region. A similar effect is observed when Cu^{2+} is chelated to acetamido and carboxyl groups intramolecularly. The four-fold helical model of Guss et al.,[159] which allows such interaction, is thus proposed

for hyaluronate in film, in acidic mixed solvent, and in Cu^{2+}-hyaluronate complex. In the fourfold helical model, there is also hydrogen bonding between the C-4 hydroxyl group of glucosamine residues and the ring oxygen of the glucuronic acid moiety. The fact that chondroitin, which differs from hyaluronic acid only in C-4 hydroxyl group configuration, shows no CD change either in aqueous-organic solvent or on complex formation with Cu^{2+} further supports the conclusion. As a matter of fact, excepting hyaluronic acid, no other glycosaminoglycans exhibit such CD changes in organic-water solvent[345] or when complexed with cupric ion.[174] This unique property of the molecule is consistent with X-ray diffraction results[164] which indicate that only hyaluronate, among all the glycosaminoglycans, can exist as a fourfold helix.

X-ray diffraction studies[159] showed that hyaluronate is highly hydrogen bonded in a solid state. Viscosity data[113] suggest that the hydrogen bonding is not significantly involved in the overall geometry of highly charged molecules (pH >>pK_a) in solution, and hence the molecule in neutral solution is probably randomly coiled. However, the possibility of helical conformation at neutral pH in a concentrated solution has not been ruled out.[114] Studies[9] suggested that hyaluronate molecules occupy a large excluded volume. This effect leads to a strong intermolecular association and probably to a helical conformation, indicated in the solid films.[159] In acidic aqueous solution, the effect of formamide indicates that hyaluronic acid is in a compact form, thus favoring intramolecular hydrogen bonding, which probably involves hydroxyl groups and ring oxygen. Park and Chakrabarti[113] thus proposed this conformation similar to a threefold helical structure;[160,161] furthermore, they[210] strongly believe that all three conformations, at higher concentration of the polymer, tend to aggregate through intermolecular interaction. The sharp decrease in optical rotation with urea,[206] the formation of viscoelastic putty[109] at pH 2.5, and the gel-like state of the molecule in mixed solvent and when complexed with Cu^{2+} are the properties of the aggregated state of the polymer.

NMR, Infrared, and Raman Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has very limited applications in the study of polysaccharides. The reason is not clear because many of these molecules are either water soluble or capable of readily forming small-particle dispersions. The general problem appears to be high molecular weight, with relatively few nonexchangeable protons in the case of most polysaccharides. Structural studies on glycosaminoglycans are complicated by insufficiency of material, difficulty in assessing purity, and a general dearth of procedures that permit selective, high-yield degradation of the polymers. Proton magnetic resonance (PMR) spectroscopy should, with suitable resolution, be useful for estimating the relative proportions of the components of a polymer from the relative intensities of resonance signals attributable to the various residues present. The technique was successfully used by Perlin and co-workers[177,211] who first reported the results of PMR spectroscopy of heparin. The 100 MHz spectra of heparins showed[211] variations among samples, but all are characterized by three signals attributed to the three main kinds of residues of heparin — *N*-sulfated glucosamine, glucuronic acid, and iduronic acid. The proportions were estimated to be equimolar. However, further measurements[177] at 220 MHz, coupled with spectral data for model compounds and with information obtained by chemical means,[212] indicated that heparins were composed mainly of 1 → 4 linked iduronic acid and *N*-sulfated glucosamine; glucuronic acid appeared to be a minor constituent. Furthermore, based on the structure of the disaccharide obtained from the enzymatic degradation of heparin, the α-L configuration was assigned to residues of L-iduronic acid and it was suggested that these uronic acids of heparin possess the 1-C conformation.

The same study[177] reported the PMR spectra of other glycosaminoglycans. Hyaluronic acid showed a poorly resolved but distinctive spectrum. The differences between chondroitin 4-sulfate, dermatan sulfate, and chondroitin 6-sulfate were evident. From the narrow spacing of the proton H-1 signal of L-iduronic acid of dermatan sulfate, Perlin et al.[177] suggested, as for heparin, an α-L configuration of this residue when it possesses the 1-C chair conformation. The general view that glycosamines are constituted basically of alternate residues of hexosamine and uronic acid was supported.

Perlin and co-workers[213,214] used ^{13}C NMR for further investigation of heparin structure. In a groundwork study,[213] they examined α- and β-D-idopyranosiduronic acids and found that in solution the α anomer adopts a conformation represented mainly by the C-1 form, and the β anomer favors this conformation exclusively. Using ^{13}C Fourier transform NMR, they then extended their analysis to heparin, as a molecular structure based on 12 carbon atoms involved in an alternating sequence consisting of 1 → 4 linked residues of α-L-idopyranosiduronic acid 2-sulfate and 2-deoxy-2-sulfamino-α-D-glucopyranosyl 6-sulfate. From the observed shifts of heparin, methyl α-D-idopyranosiduronic acid, and the model disaccharide, it was apparent that the conformation of L-iduronic acid residues in heparin may be represented most satisfactorily by the 1-C(L) conformation. The conformation of L-iduronic acid in heparin and in dermatan sulfate has been critically discussed by Perlin.[215] Although the NMR results favor 1-C conformation of L-iduronic acid in both heparin and dermatan sulfate, Perlin has not ruled out the possibility that the ring does not adopt a normal 1-C conformation, but is skewed.

The measurement of spin-spin relaxation times for the hyaluronate proton NMR studies[216] of the polymer in solution suggested some stiff segments in the domain of the polysaccharide chain. The other parts of the chain are mobile enough to give a high-resolution proton spectrum. It has been proposed that the stiff segments (ordered) are separated by more flexible, disordered domains which become conformationally mobile only on treatment with hyaluronidase or alkali. To explain the nature of this ordered structure, some intramolecular association of the segment, possibly by chain folding or looping, has been postulated. Although these authors have argued against any similarity of this structure of stiff domains with the previously proposed ordered structure,[111,202,206] the NMR results at alkaline pH do correspond with the viscosity[92] and chiroptical[203] data of hyaluronate solution at high pH values. Napier and Hadler[217] recently suggested the existence of a considerable degree of order in regions of hyaluronate at physiological concentration of Ca^{2+} from the measurements of ^{13}C NMR spectra. This ordered structure increases the translational diffusivity of lysine and glucose within such a matrix. Interestingly, the structure as well as the diffusivity is lost when the Ca^{2+} concentration is increased above 5 m*M*.

Spectral characteristics of proteoglycan aggregate and chondroitin 4-sulfate (^{13}C NMR) were compared;[218] the results indicated that the contribution to the spectrum was predominantly from glycosaminoglycan in the proteoglycan aggregates. It was suggested that protein portions of the proteoglycan aggregate, in contrast to glycosaminoglycan chains, have restricted backbone mobility and consequently a defined backbone structure. A recent report[219] presents preliminary ^{23}Na NMR studies of glycosaminoglycan solutions to obtain information on the relation between counterion binding and conformation of the polymers. The study was based on the measurements of ^{23}Na relaxation rates when an alkaline solution of glycosaminoglycan was titrated with hydrochloric acid. Although interpretation of the results seems difficult, some information was obtained on counterion binding, correlation times, and quadrupole coupling constants.

Infrared and, more recently, Raman spectroscopy have added considerably to our

knowledge of the structure of proteins and nucleic acids. These techniques have been successfully applied to cellulose, chitins, and other common homopolysaccharides and to monosaccharides.[220] Infrared studies of glycosaminoglycans have been reported previously,[221-223] but assignment of the bands was less definitive because of scarcity of well-characterized samples. Tu et al.[224] recently published the Raman and infrared spectra of hyaluronic acid and its potassium salt in the solid state. Using model compounds, such as α-methyl-D-glucoside and β-methyl-D-glucoside, they observed characteristic Raman bands at 842 cm^{-1} and 890 cm^{-1} for the α and β anomers, respectively. From a distinct band at 896 cm^{-1} and the absence of the 840 cm^{-1} band, they confirmed that hyaluronic acid contains only the β-glycosidic linkage. Bansil et al.[225] reported laser Raman spectra of chondroitin 4-sulfate, chondroitin 6-sulfate, and hyaluronic acid both in solution and in the solid state. The band assignments were made on the basis of their own and earlier infrared results (Table 5).

Sulfated and nonsulfated glycosaminoglycans can be easily distinguished from the characteristic symmetric stretching vibration of the OSO_3^- group. The frequency difference of asymmetric vibration of C-O-S linkages between chondroitin 4- and 6-sulfate has been interpreted as reflecting the equatorial configuration of the OSO_3^- group in chondroitin 6-sulfate and the axial configuration in chondroitin 4-sulfate. These preliminary Raman studies indicate considerable promise and further investigations are expected to provide a great deal of information about the structure of these macromolecules.

INTERACTION WITH IONS AND SMALL MOLECULES

The glycosaminoglycans, a class of typical anionic polyelectrolytes, exhibit the property of binding or strong interaction with small inorganic cations, with cationic molecular species, and with cationic groups or regions of macromolecules. This property has served as a basis for separation and characterization, as a method of elucidation of macromolecular conformation, and as a model of biological function of the polymers. We will review in this section the acid-base properties (H^+ binding) and the interaction of glycosaminoglycans with metal ions, cationic dyes, and quaternary ammonium salts and other biogenic amines. Included also is some discussion of the hydration properties of these molecules.

Acid-Base Properties (H^+ Binding)

Glycosaminoglycans contain carboxyl and sulfate groups, which are ionized at physiological condition. As typical polyelectrolytes, the pH-dependent molecular and interaction properties are of particular interest. Since the potential binding sites of most of these polymers are the carboxyl and the sulfate groups, reliable pK values of the groups are prerequisite for the interpretation of pH-dependent physiochemical properties of these molecules. For analysis of H^+ binding, most investigators utilized an empirical equation (Equation 8) of Katchalsky and Spitnik,[198] who extended the titration curve of a monobasic acid to a polymeric acid. Since the sulfate group is a strong acid of pK_a value below 2, the major interest in acid-base behavior has been focused on the carboxyl groups. However, the difference of pK values between the sulfate and the carboxyl groups is not large enough for successful separate titration in an aqueous media. Mathews[97,125,185] reported results of the potentiometric titration of various glycosaminoglycans. He calculated the degree of dissociation of the carboxyl group from titration results when the total sulfate and carboxyl groups were known from the weight of the sample. The data gave good agreement with Equation 8 above pH 3.5, where the sulfate group is negligibly titrated, and titration parameters (pK and n) were obtained. Recently, Park and Chakrabarti[175,197] demonstrated that pH-dependent cir-

TABLE 5

Raman Frequencies of Glycosaminoglycans and Monosaccharides[a]

Chondroitin 6-sulfate	Chondroitin 4-sulfate	Sodium hyaluronate	D-Glucuronic acid	*N*-Acetyl-galactosamine	*N*-Acetyl-glucosamine	Assignment	Ref.
			1727 (s)			C = O vibration of COOH	226
1615 (s) 1640 (sh)	1635 (s)	1620 (s) (1640 sh)		1632 (s)	1631 (s)	Amide I Shows mainly in IR	
1560 (m)	1550 (m)	1565 (m)		1590 (w)	1550 (w)	Amide II	
1459 (w,br)	1456 (w,br)	1458 (w,br)		1467 (m)	1462 (m)	CH_2 deformation	226
1413 (s)	1411 (s)	1412 (s)				COO^- symmetric	226
1377 (s)	1376 (s)	1375 (s)		1381 (s)	1383 (s)	CH_3 symmetric deformation	226
			1363 (s,br)				
1340 (s)	1341 (s)	1331 (m)		1331 (s)	1327 (s)	Amide III	227
1320 (sh)	1314 (sh)						
1298 (w)							
	1277 (w)						
1271 (m,br)	1269 (m,br)	1268 (m)	1273 (w)	1275 (s)	1266 (w)		
1237 (w)	1232 (w)		1232 (sh)			SO_3^- asymmetric stretch (strong in IR)	221,228,229
1206 (w)	1210 (w)	1206 (m)	1205 (w)		1206 (sh)		
1159 (m)	1157 (w)	1153 (m)	1155 (m)	1148 (sh)	1156 (sh)		
1120 (w)	1137 (w)	1124 (s)	1120 (vs)		1128 (vs)	$C_{(4)}$-OH, C-H, and C-OH deformation	230,231
1100 (w)	1089 (sh)	1096 (m)		1089 (s)	1087 (m)	C-OH SO^3 symmetric stretch	230,231
1062 (vs)	1079 (s)						
1050 (sh)	1050 (s)	1050 (m)	1059 (s)	1052 (sh)	1055 (s)	Partly C-OH	
1035 (sh)	1035 (sh)		1038 (w)	1023 (sh)			
1004 (sh)	1004 (sh)	1004 (sh)			998 (w)		
995 (s)	978 (m)				C-O-(S)		
975 (w,sh)	961 (sh)	970 (w)		970 (s)	964 (s)		
		960 (sh)					
937 (s)	937 (ms)	949 (s)	941 (sh)			Skeletal C-O-C linkage vibration	

TABLE 5 (continued)

Raman Frequencies of Glycosaminoglycans and Monosaccharides*

Chondroitin 6-sulfate	Chondroitin 4-sulfate	Sodium hyaluronate	D-Glucuronic acid	*N*-Acetyl-galactosamine	*N*-Acetyl-glucosamine	Assignment	Ref.
		922 (w)	915 (w)		917		
903 (sh)					902		
884 (s)	891 (m)	899 (s)	864 (m)	877 (m)		$C_{(1)}$-H deformation for β anomers	230,231
			847 (m)	825 (m)		$C_{(1)}$-H deformation for α anomer	230,231
820 (m)	853 (ms)					C-O-S (strong in IR)	221,229
780 (w)	758 (sh)		771 (m)		781 (w)		
	725 (m)	708	707 (vw)	716			
637 (w)	642 (w)	676 (w)	627 (w)	623 (m)			
578 (w)	594,547 (m)	540	572 (m)	595,527 (m)	547,525 (m)		
483 (sh)	491 (w)	490	537	486 (w)	458 (m)		
459 (w)	462 (m)	474	458 (s)			Mainly	
437 (sh)	439 (sh)	443		442 (m)		skeletal	
414 (sh)	412 (m)	413	417 (m)		409 (m)	modes	
381 (s)	375 (w)		397 (w)	375 (w)			
342 (w)	342 (w)	342 (w)	344 (w)				

* (s), strong; (m), medium; (w), weak; (sh), shoulder; (br), broad; (v), very; IR, infrared.

(From Bansil, R., Yannas, I. V., and Stanley, H. E., *Biochim. Biophys. Acta*, 541, 535, 1978. With permission.)

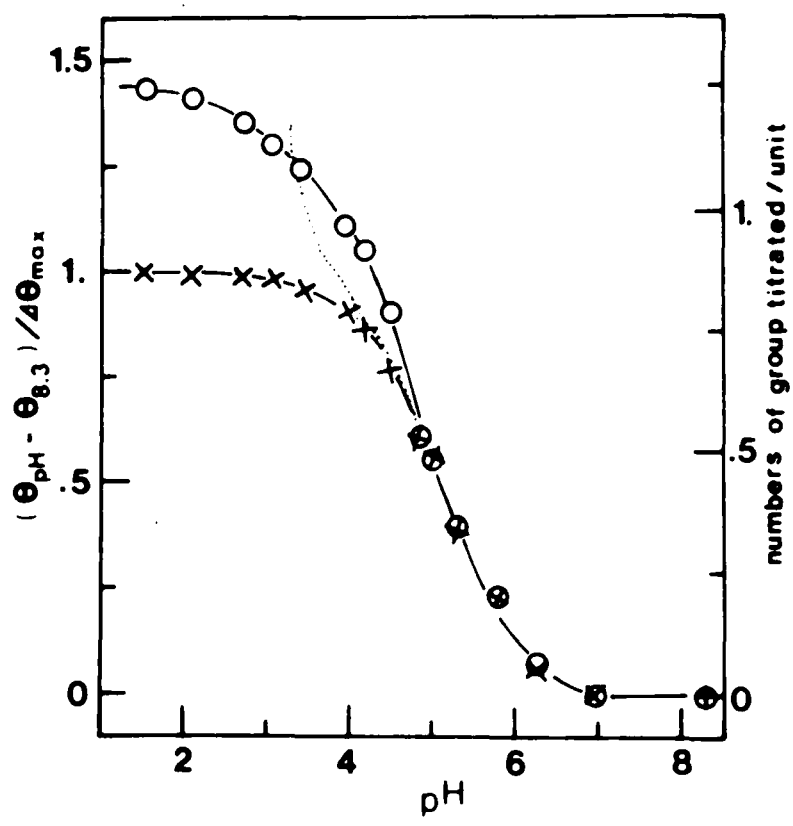


FIGURE 28. Variation of ellipticity of heparin solution at 210 (x) and 230 nm (o). $(\Delta\theta)_{max}$ was calculated from $(\theta_{1.5}-\theta_{8.3})$ for 210 nm, and from 2 $(\theta_{5.1}-\theta_{8.3})$ for 230 nm. Dotted line is the titration curve calculated by assuming that the average dimeric unit weight is 563. (From Park, J. W. and Chakrabarti, B., *Biochem. Biophys. Res. Commun.*, 78, 604, 1977. With permission.)

cular dichroic properties could be used to determine pK values of carboxyl groups. The sulfate groups had an insignificant effect in the calculation of α, even at pH about 2, so that the method could be applied even to heparin, a highly sulfated heterogeneous polymer; the pK of its carboxyl group was generally stated to be between 3 and 7 by many investigators. Figure 28 shows the pH-dependent variations of CD at 210 and 230 nm, and of potentiometric titration results of heparin.

The ellipticity changes at 210 nm form a typical titration curve and the degree of dissociation αs of iduronic acid moiety are calculated from the relationship: $\alpha = (\theta_{pH} - \theta_{8.3})/\Delta\theta_{max}$; data at 230 nm reflect titration of glucuronic acid at pH < 5. The plots of pH against log $[(1-\alpha/\alpha]$ for common glycosaminoglycans are shown in Figure 29.

To obtain the intrinsic dissociation constant, pK_o, the electrostatic potential ψ_o of the polyanion should be included in the pK-vs.-pH relationship.

$$pH = pK_a + \log \frac{\alpha}{1-\alpha} + \frac{\epsilon\psi_o}{kT} \qquad (10)$$

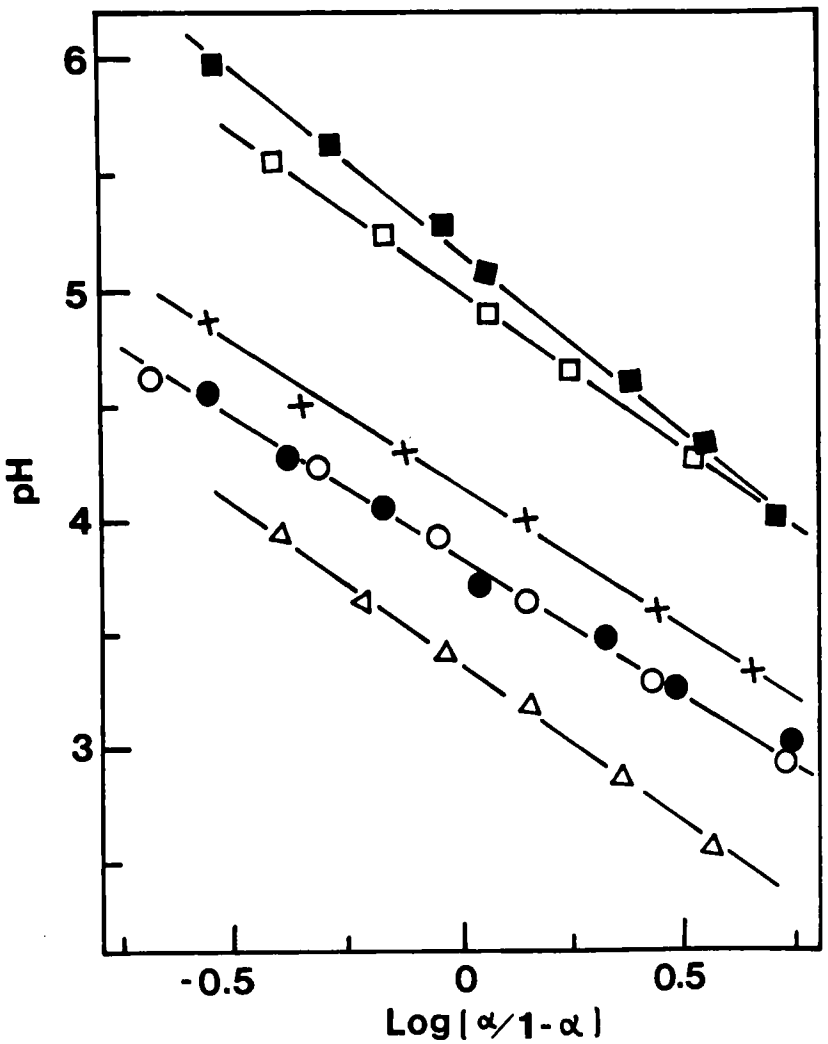


FIGURE 29. Circular dichroic titration data (heparin data is shown in Figure 28) plotted according to Equation 8 to determine pK_a values for heparin (■), dermatan sulfate (□), *N*-desulfated heparin (x), chondroitin 4-sulfate (o) and chondroitin 6-sulfate (•), and hyaluronic acid (Δ). The ellipticities employed here are measured at 210 nm for heparin and *N*-desulfated heparin, and 230 nm for the rest. (From Park, J. W. and Chakrabarti, B., *Biochim. Biophys. Acta*, 544, 667, 1978. With permission.)

Mathews[125,185] substituted 0.207 (1 + α) and 0.207 α for $\varepsilon\psi_o/kT$ for CS and DS, respectively, and for sulfate-free polymer in 0.1 M NaCl. Gilbert and Myers[232] used 0.354 α at 0.1 *M* NaCl, and 0.098 α at 1 *M* NaCl for chondroitin sulfate, and reported pK_o's of the polymers as 2.06 and 2.92 in the respective NaCl concentrations. The titration parameters of glycosaminoglycans are summarized in Table 6.

Both Mathews[97,125,185] and Park and Chakrabarti[175,197] concluded that glucuronic acid (in hyaluronic acid and chondroitin sulfate) is inherently a stronger acid than iduronic acid (in dermatan sulfate and heparin), which is also evident from Table 6. This difference in pK values of glucuronic acid and iduronic acid was clearly demonstrated from data of heparan sulfate.[175] Such difference in acid strength of the carboxyl group of C-5 epimeric uronic acids was also observed between D-mannuronic acid (pK_a = 3.38) and L-guluronic acid (pK_a = 3.65).[233] Scott[234] suggested two possibilities for

TABLE 6

Titration Parameters of Carboxyl Groups of Glycosaminoglycans[a]

Compound	NaCl conc (molar)	pK	n	pK_c	Ref.
Hyaluronic acid	0.0	3.36	1.40		175
	0.1	3.10	1.06	2.98	185
Chondroitin sulfate[b]	0.0	4.37	1.35		97,125
	0.0	3.84	1.25		175
	0.01	3.97	1.21		97,125
	0.1	3.02	1.08	2.90	185
Desulfated chondroitin	0.1	3.39	1.14	3.10	97,125,185
sulfate	0.0	3.44	1.26		175
Dermatan sulfate	0.0	5.00	1.37		175
	0.0	4.83	1.32		125
	0.01	4.46	1.23		125
	0.1	3.93	1.14	3.63	125,185
Desulfated dermatan sulfate	0.1	3.40	1.17	3.30	185
Heparin[c]	0.0	5.2	1.27		175,196
N-desulfated heparin[c]	0.0	4.20	1.26		175
N-acetylated heparin[c]	0.0	4.68	1.24		175

[a] Values are averaged from various samples.
[b] Chondroitin sulfate A and C gave the same parameters.[175,185]
[c] The parameters are those of the iduronic acid moiety.

these differences: (1) hydrogen bonding between the carboxyl group and the C-2 or C-3 hydroxyl group in D-mannuronic acid, and (2) the difference in orientation of the carboxyl group (axial for less acidic, and equatorial for stronger acid). X-ray and polarized infrared studies by Atkins et al.[235,236] revealed intramolecular hydrogen bonding between the the carboxyl group and the C-2 hydroxyl group in poly(L-guluronic acid),[236] but not in poly(D-mannuronic acid).[235]

Metal Ions

The binding or strong interactions of metal ions with glycosaminoglycans, mainly due to the anionic group, has been the subject of intensive investigations as a means of structural study and in relation to biological functions of the polymers. The control of osmotic pressure in a tissue and transport of metal ions across a tissue containing glycosaminoglycans probably are determined mainly by the interaction properties of the polymer with ions.[5] Chondroitin 4-sulfate in cartilage of proteoglycans has been shown to be the main binding site for Ca^{2+} deposition during calcification of the tissue.[237] The proposed function of cell surface glycosaminoglycans was also related to Ca^{2+} binding activities of the polymers.[238]

The metal binding studies employed various techniques to determine free ion concentration and thus bound ion, or vice versa. Among these are radiochemical analysis,[237-240] flame photometry[241] for Na^+ and K^+, EDTA titration[107] for Ca^{2+}, polarography[95] for Cu^{2+}, and selective ion electrode[96,242] for Ca^{2+}. Complexing properties of Ca^{2+} with murexide[172] and of Cu^{2+} with cuprethol[96] were also utilized. Mathews[171,173] calculated bound $Co(NH_3)_6^{3+}$ to glycosaminoglycans, and thus binding parameters, from the absorption band of the complexes in the UV range. A similar type of absorption spectral change in Cu^{2+} complexes was used to calculate binding constants of Cu^{2+} complexes by Chakrabarti and co-workers.[174,209,243] The spectral property of Cu^{2+} complexes was utilized in the determination of Ca^{2+} binding constants through competitive reaction between Cu^{2+} and Ca^{2+} with glycosaminoglycans.[243]

TABLE 7

Metal Ion Binding Constants of Glycosaminoglycans[a]

Metal ion	NaCl conc. (molar)	K_{CS}	K_{DS}/K_{CS}	K_{HP}/K_{CS}	K_{PG}/K_{CS}	Ref.
Ca^{2+}	0.15	16.3	1.5	8.3	0.94	172
Ca^{2+}	0.1	55	2.2	6.7	—	243
Ca^{2+}	0.1[b]	65	—	—	1.0[c]	107
Cu^{2+}	0.1	57	4.0	16.5	—	243
$Co(NH_3)_6^{3+}$	0.1[d]	1500	6.0	11	—	171,173

[a] CS, chondroitin sulfate; DS, dermatan sulfate; HP, heparin; PG, proteoglycan.
[b] Measured at 6°C.
[c] Ratio for aggregated proteoglycans was 1.7.
[d] CS data were taken from 0.1 *M* $NaClO_4$. Ratio of binding constants were calculated from several sets of data at same ionic strength.

Dustone[239,240] investigated ion exchange reactions between glycosaminoglycans and various monovalent and divalent ions. With the exception of heparin, all glycosaminoglycans showed higher affinity for Na^+ than for K^+. The reverse order of affinity for heparin was also reported by Salminen and Luomanmäki.[241] However, divalent binding was dependent on the glycosaminoglycans, probably due to the difference in the structure of the polymers: $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ for chondroitin 4-sulfate, cartilage, and dermatan sulfate; $Mg^{2+} < Sr^{2+} < Ba^{2+} < Ca^{2+}$ for heparin; $Mg^{2+} < Ca^{2+} = Sr^{2+} < Ba^{2+}$ for heparan sulfate.

Orders of binding among glycosaminoglycans were established by Mathews[171,173] for $Co(NH_3)_6^{3+}$, by Buddeke and Drzenick[172] for Ca^{2+}, and by Park et al.[243] for Cu^{2+} and Ca^{2+}. The results are summarized in Table 7.

Strong ionic strength dependence for metal ion binding constants were shown by Mathews,[171,173] Park et al.,[243] and MacGregor and Bowness.[107] Such strong ionic strength dependence and parallelism between binding constants of different cations suggest that similar electrostatic forces may be predominating factors for these cation bindings. The higher interaction properties of iduronic acid-containing glycosaminoglycans (heparin and dermatan sulfate) over glucuronic acid-containing ones (hyaluronic acid and chondroitin sulfate) were also exhibited in the associations with H^+ and other cationic macromolecules, which could also be accounted for mainly as electrostatic interaction.

Interaction of Cu^{2+} with heparin was extensively investigated by Stivala's group in relation to anticoagulant and Cu^{2+} binding activities of the polymer. The group reported variations of Cu^{2+} binding of different molecular weight fractions of heparin as a function of pH, and showed that both the Cu^{2+} binding and anticoagulant activities of heparin, denoted as ϕ by the authors, were closely related to the molecular weight (M) of the polymer, obeying an empirical equation: $1/\phi = a + b/M$, where a and b are constant.[95] Such correlation between Cu^{2+} binding and biological activities of heparin was also demonstrated from the acid-catalyzed hydrolysis of the polymer; *N*-desulfation of heparin was accompanied by loss of bound Cu^{2+}, which was followed by reduction in its biological activity. The $Co(NH_3)_6^{3+}$ binding data of Mathews[173] also indicated association of anticoagulant activity with high binding affinity for $Co(NH_3)_6^{3+}$.

Both sulfate and carboxyl groups of a glycosaminoglycan can be binding sites for cations. Lages and Stivala[96] reported that the heparin fraction of molecular weight 13,800 bound cooperatively a maximum of 21 to 24 Cu^{2+} in two different classes of

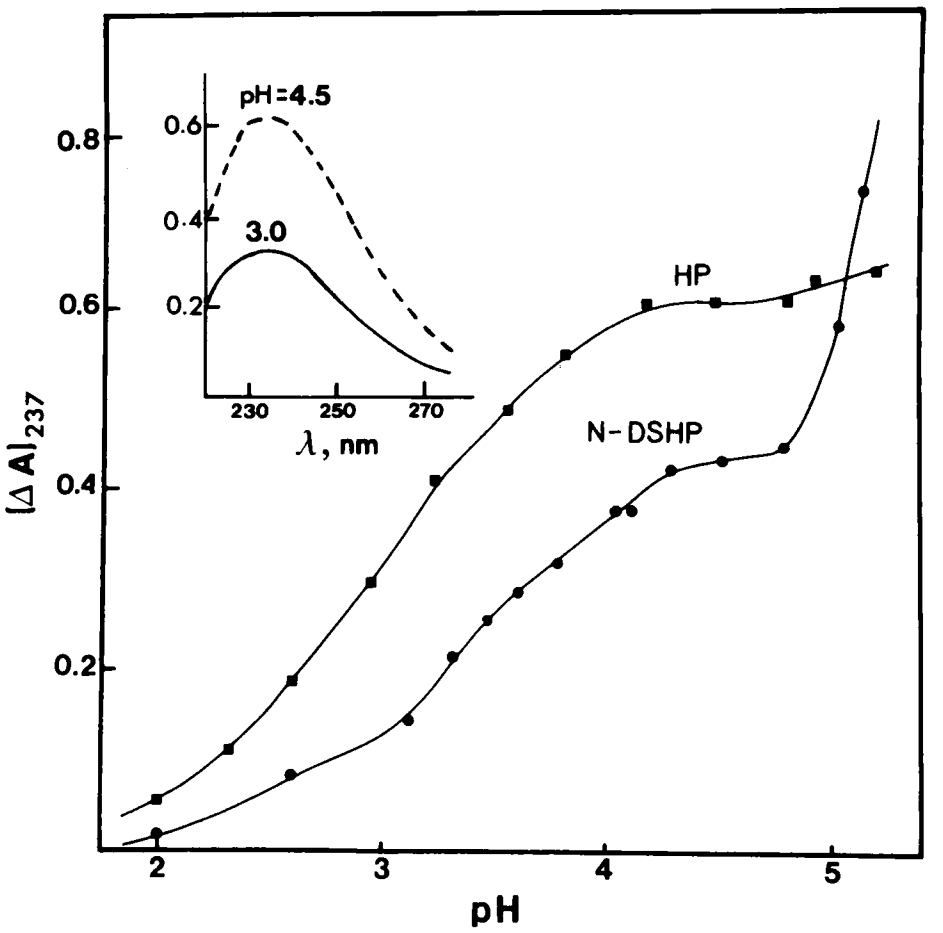


FIGURE 30. Variation of $[\Delta A]_{237}$, the difference absorption spectra between a mixture and unmixed components, of 0.4 mg/ml heparin (HP) and *N*-desulfated heparin (N-DSHP) with pH in the presence of 5×10^{-3} *M* Cu^{2+}. Inset shows difference absorption spectra of heparin at two pH values. (From Mukherjee, D. C., Park, J. W., and Chakrabarti, B., *Arch. Biochem. Biophys.*, 191, 393, 1978. With permission.)

binding sites — one containing 3 to 4 binding sites, and the other containing 20 to 21 sites which did not bind in acidic media. However, they did not rule out the possibility that the two binding sites are identical. The close relationship between the number of total carboxyl groups of the polymer and the number of binding sites, and the pH dependence of binding suggest that the Cu^{2+} binding sites are indeed ionized carboxyl groups. The acid-catalyzed hydrolysis of heparin and heparin-Cu^{2+} complex showed that both first-order rate constants and activation energies of the two systems are in good agreement with each other. Further support on binding sites of glycosaminoglycans was provided by Mukherjee et al.[174] They studied the characteristic 237 nm absorption band of Cu^{2+} complexes of glycosaminoglycans as a function of pH (Figure 30) and showed that a simple competition reaction between H^+ and Cu^{2+} for a common binding site, carboxyl group, can satisfactorily explain the pH dependence of the absorption band.[96] In the wide range of Cu^{2+} to glycosaminoglycan ratio, no evidence of significant binding of Cu^{2+} to sulfate was shown.

Analysis of pH-dependent absorption data shown in Figure 30 with the independently measured p*K* of the carboxyl group gave a binding constant of 1×10^4 and $2 \times$

10^2 M^{-1} for Cu^{2+} with heparin and *N*-desulfated heparin, respectively, in the presence of 5×10^{-3} M Cu^{2+}. Mathews[171,173] also showed a lower $Co(NH_3)_6^{3+}$ binding constant for acetylated heparin and a higher one for sulfated heparin in comparison with that of heparin. Such changes in metal ion binding affinity, which are in parallel with the p*K* of the carboxyl group,[96] with the chemical modification of heparin reflect the charge density of the macroanions: heparin has a negative charge in *N*-sulfate, whereas acetylated heparin and *N*-desulfated heparin have a zero and a positive charge, respectively, in the group derived from *N*-sulfate.

The one-to-one stoichiometry of Cu^{2+} glycosaminoglycan complexes and the explanation of insignificant Cu^{2+} binding to the sulfate group does not rule out the possibility of involvement of sulfate groups in the complex formation. Chakrabarti and co-workers[115,174] have shown marked CD changes in heparin, desulfated heparin (pH > 5), heparan sulfate, hyaluronic acid (pH > 6), and dermatan sulfate in the presence of Cu^{2+}. However, no CD changes were observed in chondroitin, chondroitin sulfate, desulfated heparin (pH < 5), and hyaluronic acid (pH < 6). The CD changes in the first group of glycosaminoglycans were ascribed to asymmetry of the chelating ring arising from strong interaction of sulfate, free amine, or deprotonated amine with carboxyl-Cu^{2+} ion pairs (or complexes), which shows a charge-transfer band at 237 nm in the absorption spectra. This explanation of an extrinsic CD band in Cu^{2+} complexes contradicted the attribution of the CD to a conformational change of heparin by Chung and Ellerton,[106] but supported the viewpoint of Lages and Stivala[96] who concluded, from viscosity and sedimentation studies of heparin as a function of Cu^{2+} concentration, that there were no major changes in the solution conformation of the polymer. The higher ratios K_{DS}/K_{CS} and K_{HP}/K_{CS} for Cu^{2+} and $Co(NH_3)_6^{+3}$, in comparison with Ca^{2+} results (Table 7), suggest that the involvement of the sulfate group in complex formation also affects the stability of the complexes.

Tanaka[100] studied the interaction between cations and chondroitin sulfate by measuring electric conductivity, and reported no detectable difference in the ion binding properties of chondroitin 4-sulfate and 6-sulfate. He suggested that the interaction of Ca^{2+} with chondroitin sulfate might not be ascribed solely to electrostatic forces. Vannucchi and co-workers[238] compared relative Ca^{2+} binding capacity, not affinity, of glycosaminoglycans (HA:CS:DS:HP = 1:1.6:1.8:2.8) by equilibrium dialysis. Localization of various metal ions including ferrous and ferric, by external glycosaminoglycan surface coat encompassing the neurons, was reported by Garcia-Segura.[244]

Exclusion of phosphate ion by chondroitin sulfate and proteoglycans was demonstrated by MacGregor and Bowness.[107] This phenomenon was interpreted as a Gibbs-Donnan or repulsion effect of charges of the polymers. Németh-Csoka[245] showed accumulation of citrate and phosphate ions by collagen, and decrease of bound anions with addition of chondroitin sulfate.

Some differences of the solid-state molecular conformation between the sodium and calcium salts of hyaluronic acid,[162] chondroitin 4-sulfate,[167] and heparin and heparan sulfate[165] were reported. The detailed results are presented in the section on X-ray diffraction studies.

The calcium binding of proteoglycans and comparison of the data with that of chondroitin sulfate were investigated by several groups after the report of Boyd and Newman,[237] who showed that chondroitin 4-sulfate in costal cartilage is mainly responsible for the cation binding of the tissue. Woodward and Davidson[242] reported that two sulfate groups from different chains of chondroitin 4-sulfate from proteoglycan bound to Ca^{2+} so tightly that the cation could not be replaced by sodium ion, whereas isolated chondroitin sulfate itself had little affinity for Ca^{2+}. This finding contradicted other observations by Buddeke and Drzenick,[172] by Mathews,[246] and by MacGregor and

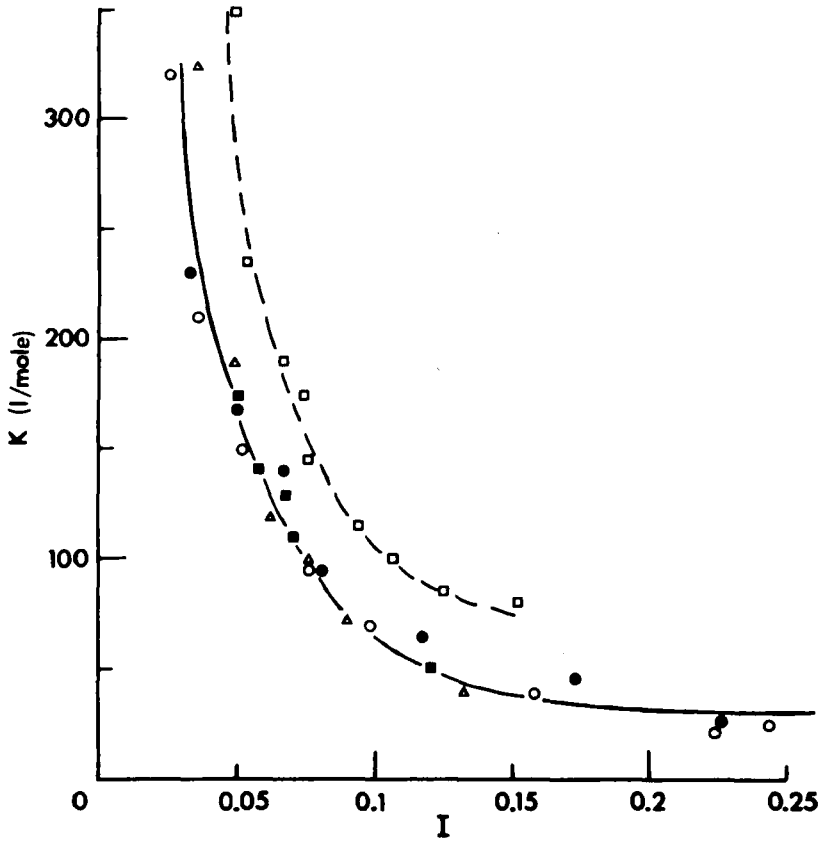


FIGURE 31. Variations of Ca^{2+}-polymer complex formation constant (K) with nonpolymer ionic strength (I). (•) chondroitin 4-sulfate (collidine-HCl, pH 7.0), (o) Na^+-proteoglycan (collidine-HCl, pH 7.0), (Δ) Ca^{2+}-proteoglycan (collidine HCl, pH 7.0), (□) aggregated Ca^{2+}-proteoglycan (Tris, pH 7.1), (■) Na^+proteoglycan (Tris, pH 7.1). (From *Can. J. Biochem.*, 49, 417, 1971. With permission of the National Research Council of Canada.)

Bowness,[107] who did not observe any significant difference in Ca^{2+} binding affinity between proteoglycan and isolated chondroitin sulfate. The results of MacGregor and Bowness[107] as a function of ionic strength are shown in Figure 31.

However, their results indicated significantly greater binding affinity for Ca^{2+} in an aggregated proteoglycan: the binding affinity decreased after disaggregation. It would be interesting to compare this finding to the observations that an aggregated proteoglycan delayed the sedimentation of freshly precipitated calcium phosphate[247] and inhibited the growth of calcium hydroxyapatite crystal.[248] The calculated Ca^{2+} binding constant[107,243] for chondroitin sulfate, 50 to 70 M^{-1} in 0.1 *M* NaCl, could not explain the observed near-equivalence of 50% Ca^{2+} content of cartilage. The higher binding affinity of aggregated proteoglycan can partly explain the result. However, the theoretical work of Manning[249] is worth noting. He predicted condensation of divalent ions onto the anionic polyelectrolytes even in the presence of a large excess of monovalent ions.

Recently, Vannucchi et al.[238] showed a good correlation between the Ca^{2+} content of cells and the amount of cell-coat glycosaminoglycans; growing 3T3 cells contain less Ca^{2+}, which is in parallel with decreased sulfated glycosaminoglycans of the cells. They suggested that *N*-sulfated glycosaminoglycans of the cell coat could exert their negative effect on growth via their strong sorption of Ca^{2+} ions. This is in good agree-

ment with reports of decreased dermatan sulfate and heparan sulfate content of neonate and tumoral tissues, in comparison to normal adult tissues, and with the suggestion of a possible role of sulfated glycosaminoglycans in cell recognition and adhesiveness.[17,48]

Silicon has been claimed to be an integral constituent of glycosaminoglycans.[250-252] Schwarz[250] showed that glycosaminoglycans from various sources are bound to silicon. He suggested that silicon may function as a biological crosslinking agent and contribute to the architecture and resilience of connective tissue. In vivo experiments with rats by Carlisle[252] indicated silicon involvement in glycosaminoglycan synthesis.

Cationic Dyes Binding

The interaction properties of cationic dyes with glycosaminoglycans have been studied for various reasons. The marked changes of spectral properties of dyes upon binding to polyanionic molecules, metachromacy, has long been utilized in histological staining procedures to determine the location of glycosaminoglycans and other polyanionic materials in cells and tissues. The Cotton effect induced by some metachromatic dyes was used to elucidate molecular conformations of glycosaminoglycans. The technique was first introduced for polypeptides.[253,254] Glycosaminoglycans do not have a chromophore in the wavelength region above 250 nm and thus spectral properties of dye-glycosaminoglycan systems in the region arise from either free or bound dyes. Since most dyes used in those experiments are achiral molecules, chiroptical properties of the system, circular dichroism and optical rotatory dispersion, can be assigned to those of bound dyes in the asymmetric environment of host glycosaminoglycans. Earlier results on this subject can be found in a review by Stone.[3]

Tinoco[255] proposed a relationship between the doublet CD band of induced Cotton effect and the chirality of helix of the polymer molecule: a negative peak at the high frequency and an equal positive peak at the lower frequency for the left-handed helical polymer, and the converse for the right-handed helix. Stone[200] reported induced Cotton effects of glycosaminoglycans with methylene blue showing negative doublet CD bands for heparin, heparan sulfate, and keratan sulfate, and positive ones for chondroitin sulfate and dermatan sulfate. In the analogy with protein, she attributed the doublet CD bands to the helical conformations of the polymers.

In contrast to the methylene blue results, acridine orange (AO) complexes with heparin and with chondroitin 6-sulfate showed induced ORD of the same shape and sign. Stone[3] interpreted this as evidence of a nonspecific binding property of acridine orange among the various acid polysaccharides. Németh-Csoka et al.[256] reported that the sign and magnitude of intrinsic and extrinsic Cotton effects of methylene blue complexes of sulfated glycosaminoglycans varied with the sulfate content of the polymers, and suggested that the extent of sulfation rather than the glycosidic structure might determine the chiroptical and functional properties of the glycosaminoglycans. Chakrabarti and Balazs[202] attributed a symmetric doublet CD band of an hyaluronate-AO system of 2.5×10^{-5} *M* AO concentration to an indication of left-handed chirality of the polymer molecule, as suggested from X-ray studies. However, the system displayed a single CD band when the AO concentration was 10^{-4} *M*, which was explained as dye-dye interaction. In support of the helical structure of hyaluronic acid, they reported that heat-denatured hyaluronic acid, when rapidly cooled, did not show an induced Cotton effect, but when it was slowly cooled, it did exhibit some induced Cotton effect. Effect of temperature on the induced ORD of dye-glycosaminoglycans was studied by Nakajima and Matsumura.[257] They showed that, at room temperature and neutral pH, toluidine blue and methylene blue complexes of hyaluronic acid displayed a Cotton effect of opposite sign to those of chondroitin sulfate complexes, despite the structural simi-

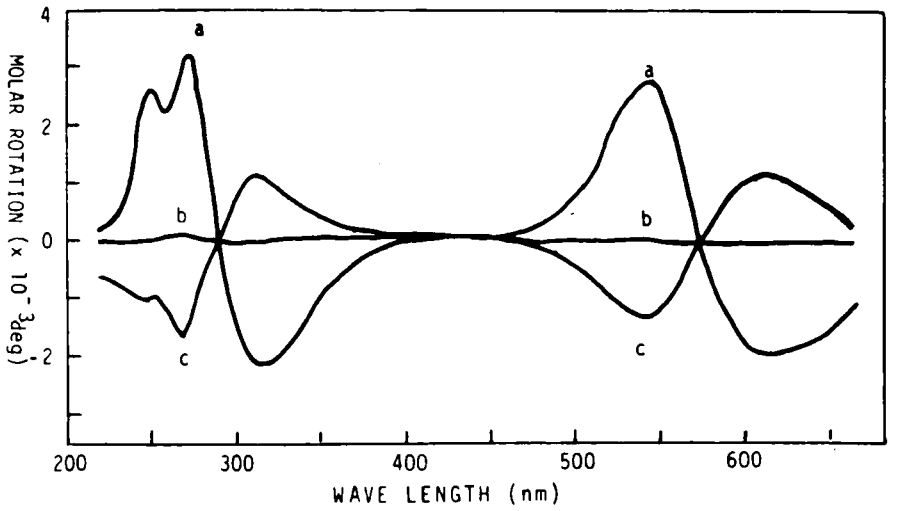


FIGURE 32. Induced Cotton effect of chondroitin 4-sulfate/toluidine blue at pH 5.4 showing inversion of optical rotatory dispersion with temperature: (a) 22°C, (b) 72°C, (c) 76°C. Concentration of dye is 5×10^{-5} *M*; polymer to dye ratio is 0.8. Similar inversion was also reported for chondroitin 6-sulfate/toluidine blue complex. (From Nakajima, K. and Matsumura, G., *Biopolymers*, 12, 2539, 1973. With permission.)

larity between the two polymers. Gradual decrease in the metachromatic shift in the absorption spectra and in the induced ORD with increasing temperature was observed for both complexes. However, at higher temperature, toluidine blue complexes of both chondroitin 4-sulfate and chondroitin 6-sulfate exhibited inversion of sign of the Cotton effect, as shown in Figure 32.

The Cotton effect of hyaluronic acid-toluidine blue diminished at pH 3, at room temperature; that of chondroitin 6-sulfate complex reversed its sign on acidification to pH 3. These workers concluded that the sign of the induced Cotton effect did not indicate the entire conformation of glycosaminoglycans.

Salter and co-workers[258,259] recognized two types of AO complexes of dermatan sulfate and chondroitin sulfate, based on their solubility in water, which differed in their absorption and circular dichroic spectra. The CD spectra of the soluble chondroitin sulfate-AO and dermatan sulfate-AO complexes were similar, but the spectra of the aggregated and partially soluble complexes were almost mirror images of each other (Figures 33, 34).

Their report also indicated that heat treatment of glycosaminoglycans or their dye mixtures had no effect on the CD properties of the complexes of high P/D ratio,[258] but produced a sharp drop in induced CD for complexes of smaller P/D ratio. Such temperature dependence was ascribed to a cooperative change in the structure of the complex, which might differ from the intrinsic structure of the glycosaminoglycan itself in dilute solution. The complex CD properties and the temperature effects on the properties of the aggregate complexes were attributed to a liquid-crystal-type supermolecular ordering of complexes, which could be reorganized to a more stable form at high temperature.[259]

Studies on the binding of dyes to glycosaminoglycans by absorption and fluorescence spectroscopy require differentiation of spectral characteristics of free and bound dyes. West el al.[260] employed membrane filtration technique to remove free dyes from a mixed solution of AO and dermatan sulfate, and showed that the species emitting at 540 nm is due only to free dye whereas the 660 nm-emitting species was due to bound dye (Figure 35).

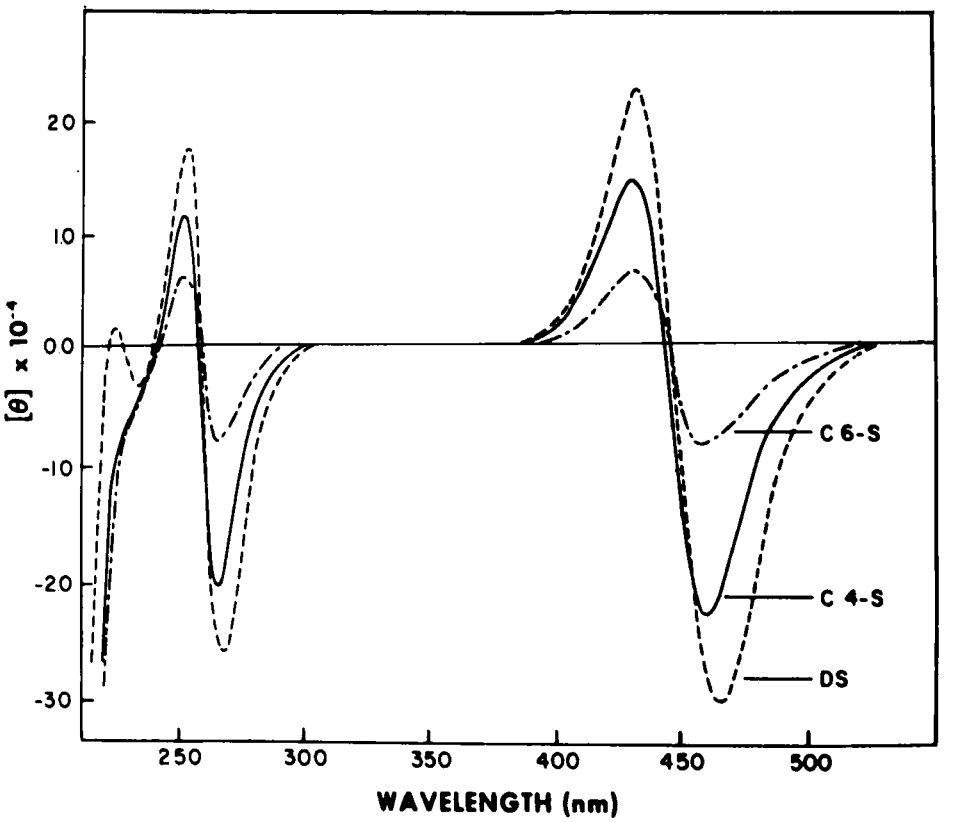


FIGURE 33. CD spectra of soluble acridine orange complexes of glycosaminoglycans. Dye concentration is 1×10^{-5} *M*: polymer to dye ratios (P/D) are 11 for dermatan sulfate (DS) and 10 for both chondroitin 4-sulfate (C 4-S) and chondroitin 6-sulfate (C 6-S). Compare this data with Figure 34 to see the sensitivity of induced Cotton effect on P/D. (From Salter, M. K., Rippon, W. B., and Abramson, E. W., *Biopolymers*, 15, 1213, 1976. With permission.)

The same group of investigators subsequently determined the thermodynamic parameters of the AO-dermatan sulfate complex.[261] Data indicated that dermatan sulfate bound 2.3 ± 0.3 molecule of AO per disaccharide unit with a cooperative binding constant of 4.9 to 6.0×10^5 M^{-1}, which corresponds to free energy of 7.74 to 7.86 kcal/mole. They also reported apparent increase in the cooperativity parameter with increasing P/D ratio.

It seems that the elucidation of molecular conformation of the glycosaminoglycans based on induced Cotton effects of dyes, which are sensitive to various experimental factors, might be an oversimplification of the complex system. The implied helical structure of the polymer in dye complexes may not be the intrinsic conformation of an isolated glycosaminoglycan molecule, but an induced one resulting from strong interaction with dyes — if, indeed, the polymer does have a helical structure in the complex.

Quaternary Ammonium Salts and Other Amines

A typical polyanionic property of glycosaminoglycans also appears as formation of water-insoluble complexes with quaternary ammonium compounds, such as cetylpyridinium. The insoluble complexes are dissociated and dissolved by inorganic salts at critical concentration depending largely on the charge density of the polymers.[262] These

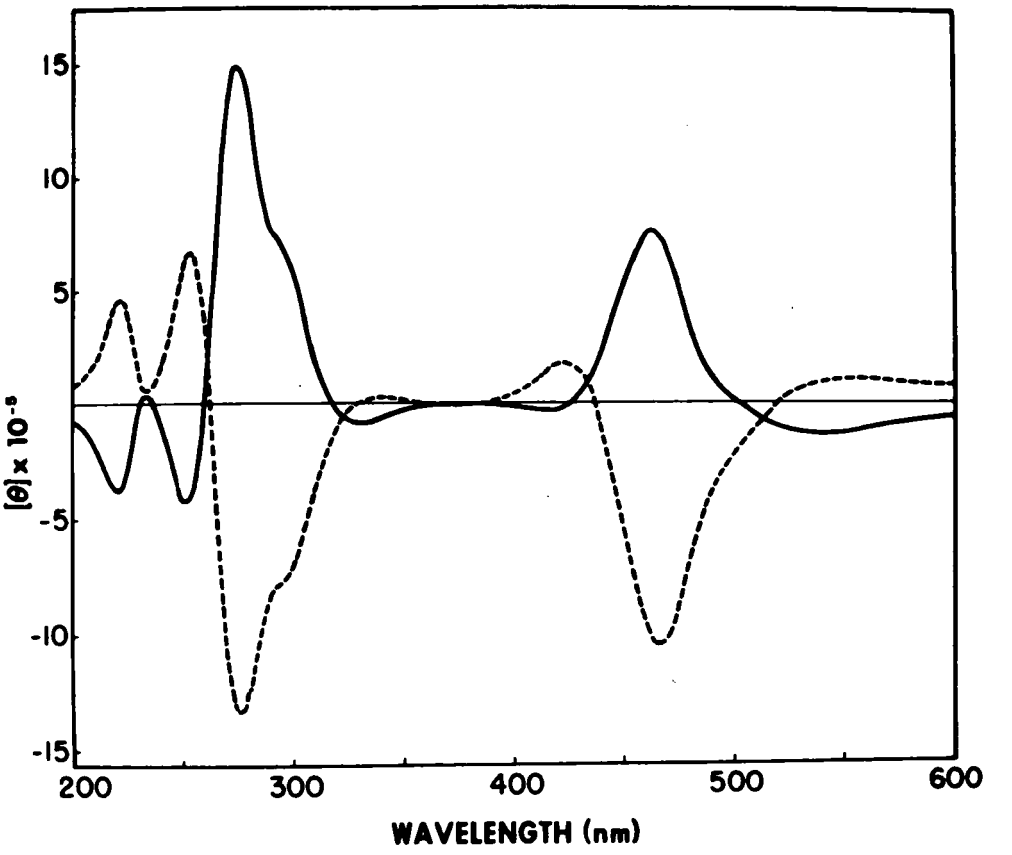


FIGURE 34. CD spectra of aggregated chondroitin 4-sulfate/acridine orange (broken line) and dermatan sulfate/acridine orange (solid line) complexes. Dye concentration is 5×10^{-5} *M*; P/D is 1. (From Salter, M. K., Abrahamson, E. W., and Rippon, W. B., *Biopolymers*, 15, 1251, 1976. With permission.)

phenomena have been used in recovery, quantitation, and fractionation of glycosaminoglycans.[13,19,262]

Jennings and Hurst[263] demonstrated that the complexes could be partitioned between aqueous inorganic salt and butanol phases, showing a sharp transition in solubility. These observations were extended to various phase compositions such as the type of inorganic salt, the type of quaternary ammonium salt, and the specific alcohol.[264] The effects of inorganic salts are shown in Figure 36.

Two series of fractionated chondroitin sulfate, which differed in mol wt and sulfate content, were isolated by this technique, showing that the degree of sulfation was a major determinant of the partition properties.[265] However, the partition behavior was shown to be insensitive to molecular weight in excess of 12,000. From this result, the investigators argued against the critical electrolyte model of Scott,[262] but suggested that solvent-solute interaction is reflected in the partition phenomenon.

Heparin is a major constituent of the mast cell granule, and this negatively charged macromolecule was assumed to be the site of histamine binding within the cell. Keller[266] reported weak binding of histamine and serotonin with heparin in aqueous solutions at pH 5 to 5.5: increasing pH or salt concentration dissolved the amine-heparin complexes. The binding of histamine with heparin was also demonstrated by Kobayashi[267] and Barlow.[268] The changes in optical rotatory dispersion of heparin on the binding of histamine and serotonin were shown by Stone.[195] Uvnäs et al.[269] proposed that his-

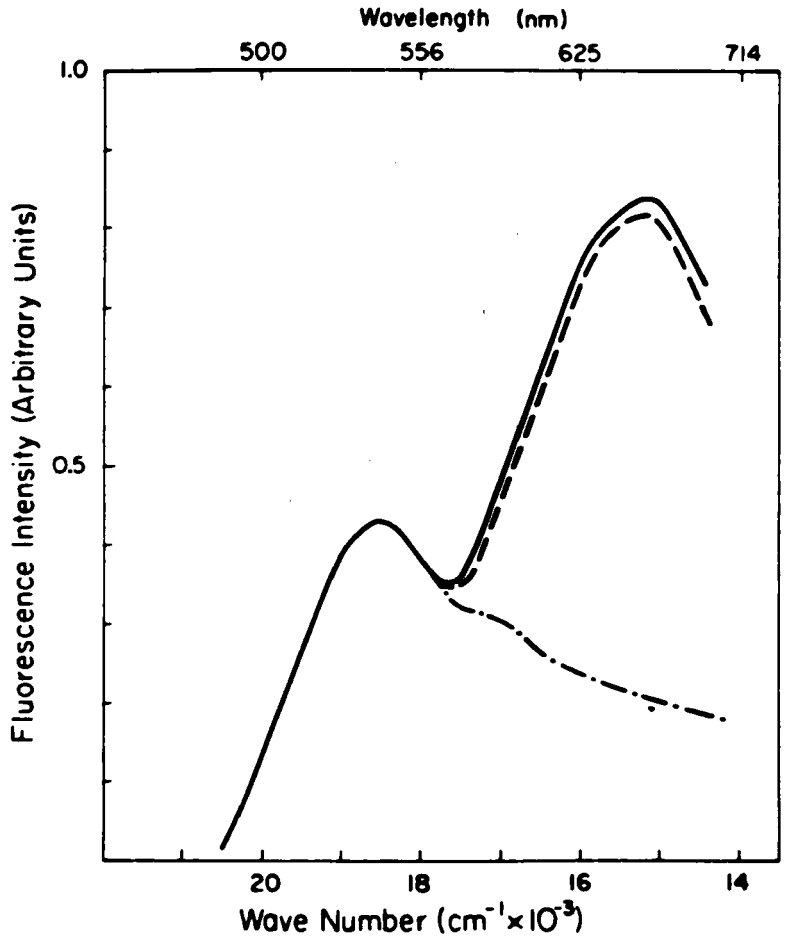


FIGURE 35. Rapidly recorded, corrected fluorescence emission spectra of acridine orange-dermatan sulfate solutions after a small aliquot was passed through a Nuclepore filter (0.03-nm pore size). Total volume of solution, 8 m*l*. Original solution (—), filtrate (.—.); solution remaining in syringe (— —). The 540-nm peak is present in all three spectra. Total AO concentration of the original solution, $[AO]_t = 1 \times 10^{-5}$ *M*, P/D ratio = 2, in 10^{-3} *M* citrate/phosphate buffer (pH = 7.0), t ≈ 24°C. Excitation was at 436 nm. Emission band at 660 nm was attributed to bound dye. (From West, S., Hurst, R. E., and Menter, J. M., *Biopolymers*, 16, 685, 1977. With permission.)

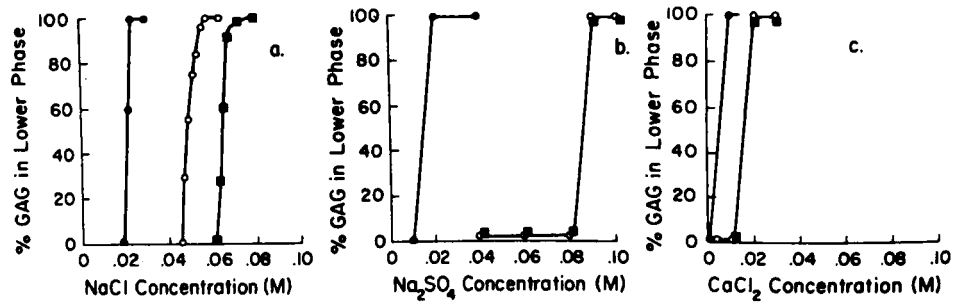


FIGURE 36. The partition of glycosaminoglycan-hexadecyl pyridinium (HPC) complexes between aqueous salt solutions and 1-butanol phases at 1% (w/v) HPC as a function of the salt concentration. (•) hyaluronate, (o) dermatan sulfate, (□) heparin. (From Hurst, R. E. and Sheng, J. Y., *Biochim. Biophys. Acta*, 444, 75, 1976. With permission.)

tamine bound to the carboxyl groups of granule protein, rather than to the negative site of heparin which was assumed to interact with the positively charged groups of protein. However, from the analysis of dye binding of mast cell granules, Lagunoff[270] suggested that the *N*-sulfate, carboxyl, and *O*-sulfate groups were available for binding with small-molecular-weight cations such as dyes and histamine.

It was shown[271,272] that heparin could be fractionated by isoelectric focusing into at least 21 components depending on mol wt, with PI values in the pH range 3.2 to 4.2. Righetti and Gianazza[273,274] demonstrated that the basis for such fractionation by isoelectric focusing is, in fact, a strong interaction between heparin and various amphoteric species in the ampholine mixture.

Hydration

Glycosaminoglycans are believed to be involved in the retention of water in connective tissue. The terms *water binding* and *hydration* have been commonly used for the interaction with water, but these are ambiguous and require careful evaluation, as pointed out by Ogston.[275]

Sorption isotherms of water on glycosaminoglycans yielded B.E.T. II-type* multilayer curves.[276] The amount of water vapor absorbed at a given humidity was related to the chemical structure and the physical state of aggregation. The sorptive capacities of sulfated glycosaminoglycans were in the order: heparin > chondroitin 6-sulfate > dermatan sulfate > chondroitin 4-sulfate, at low vapor pressure, but the order of heparin and chondroitin 6-sulfate was reversed at high vapor pressure. Bettelheim and Ehrlich[276] reported the number of water molecules per disaccharide unit required to form a monolayer from B.E.T.-type isotherms: 1.2 for chondroitin 4-sulfate, 1.9 for dermatan sulfate, 2.2 for chondroitin 6-sulfate, and 2.4 for heparin.

Bettelheim and Plessy[277] studied water vapor sorption to chondroitin 4-sulfate, keratan sulfate, and proteoglycans of the polysaccharides from bovine cornea, and found that keratan sulfate and proteoglycan containing predominantly keratan sulfate chains exhibited a large amount of bound water. Topographic distribution of hexosamines in bovine cornea was investigated and the distribution was related to hydration properties of proteoglycans.[278] Higher swelling values were observed by Mathews and Decker[279] for shark cartilage than for bovine and sturgeon cartilage; these were attributed principally to a higher ratio of glycosaminoglycan to collagen. Sweet and co-workers[280] reported higher content of water in articulatory than in the corresponding growing zone of immature cartilage, but less water in both zones in the areas of maximum contact. They reported, however, more hyaluronate and much more keratan sulfate in the articulating areas of maximum contact than in the minimum contact areas.

It is well known that an electrical potential develops between the ends of a column of hyaluronate in a U-tube displaced by gentle pressure.[23,24] Barrett[24] demonstrated the release of bound water with applied pressure, and interpreted the mechano-electrical phenomenon as an entropy-driven process.

The interaction between glycosaminoglycans and water also affects the water activity and appeared as flow resistance in polysaccharide networks. Readers interested in these properties are referred to a recent review by Comper and Laurent.[5]

INTERACTION WITH MACROMOLECULES

The glycosaminoglycans are found in various tissues, usually with other macromolecules, especially proteins. It is highly likely that the biological role of glycosamino-

* Abbreviation from Brunauer, Emmett, and Teller model.[347]

glycans is closely related to the interaction properties with other macromolecules. After a review of these interactions, the phenomenon of steric exclusion of a glycosaminoglycan matrix is also presented.

Synthetic Polypeptides, Noncollagenous Proteins, Lipoproteins, and Proteoglycans

The interaction between cationic polypeptides and glycosaminoglycans has been extensively investigated as a model for connective tissue. These interactions are mostly electrostatic as they are known to be sensitive to pH and ionic strength. Gelman and co-workers[281,287] reported on CD studies of interactions between glycosaminoglycans and the cationic polypeptides poly-L-arginine (PLA) and poly-L-lysine (PLL). The results were summarized in a recent article.[288] Their data showed that both PLA and PLL adopt α-helical conformation at neutral pH in the presence of glycosaminoglycans rather than the normal charged random-coil form observed in the absence of the polysaccharides. The typical CD spectra demonstrating the conformational change of PLL are shown in Figure 37.

The induced α-helical conformation of polypeptides is disrupted at higher temperature. There is a sharp transition temperature range, above which a spectrum of a mixture is identical to the simple sum of the ellipticities of the components. The stoichiometry and melting temperature (defined as the midpoint of the plots of ellipticity at 222 nm against temperature) are summarized in Table 8. The six common glycosaminoglycans can be placed in order of increasing strength of interaction (stability of complex): hyaluronic acid < chondroitin-4-sulfate < heparan sulfate < chondroitin 6-sulfate < keratan sulfate < dermatan sulfate < heparin.

The effects of ionic strength and pH on the interaction between glycosaminoglycans and polypeptides were reported by Schodt et al.,[287] who showed breakdown of the α-helix as the ionic strength of the media was increased. The order of increasing strength of interaction, based on the ionic strength at which disruption occurred, is: chondroitin 6-sulfate < hyaluronic acid < chondroitin 4-sulfate < heparin < dermatan sulfate. However, the α-helix of PLA complexes do not break down as the ionic strength is increased, indicating a stronger interaction for PLA. The pH-induced transition of complexes that occurs at pH 2.4 to 3.8 and near pH 11 could be related to protonation of the carboxyl group of glycosaminoglycans and deprotonation of the amino group of polypeptides, respectively. The investigators suggested that the interaction may not be purely electrostatic, but that conformation of the glycosaminoglycans might influence the interaction. This suggestion was based on the disagreement of pK_a's of the carboxyl group of glycosaminoglycans and transition pHs. However, the pK_a of the carboxyl group of glycosaminoglycans in complex may differ considerably from values obtained in the absence of the strongly interacting cationic polypeptides.

Stone and Epstein[289] reported CD studies on the interactions between heparin and L-lysine copolymers, poly-(L-lysine to L-tyrosine, 1 to 1) and poly-(L-lysine to phenylalanine, 1.4 to 1). They showed that the former copolymer shows a poly-(L-lysine)-like CD spectrum, whereas the latter displays an α-helical structure. Their data indicated that the change in CD spectra increased with the addition of heparin until the ratio of positive to negative charge is about one. They claimed that such strong interaction can provide a basis for the use of heparin as an analytical probe for the measurement of charge and conformation potential in basic proteins isolated from biological sources. The induced conformational change of cationic polypeptides is not unique to glycosaminoglycans. Other polyanionic macromolecules such as polygalactouronate, kappa-carrageenan, and polyphosphate also promote the α-helical conformation in PLL.[290]

Strong interaction of glycosaminoglycans with basic natural proteins has also been shown. Precipitation of serum albumin in acidic hyaluronate media, mucin clot for-

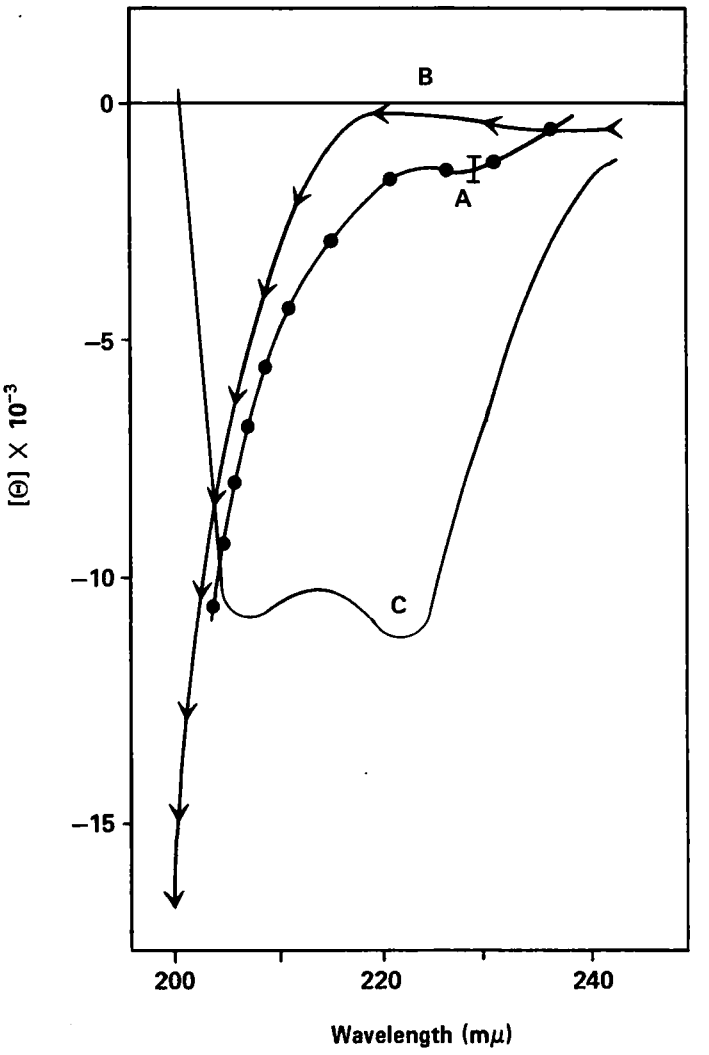


FIGURE 37. (A) CD spectrum of a 1:1 mixture of poly-L-lysine (PLL) and chondroitin 4-sulfate, (B) calculated spectrum predicted for a 1:1 non-interacting mixture of PLL and chondroitin 4-sulfate, (C) CD spectrum of 1:1 mixture of PLL and chondroitin 6-sulfate. All are for solution in water at neutral pH. B is a typical spectrum of polypeptide in random conformation; C is that of α-helix. Ellipticities of glycosaminoglycans are very small near 220 nm compared to those of polypeptides. (From Gelman, R. A. and Blackwell, J., *Biopolymers*, 12, 1959, 1973. With permission.)

mation in synovial fluid on the addition of acetic acid, and removal of lysozyme by chondroitin sulfate from low salt solutions can be considered as examples of strong interaction between glycosaminoglycans and cationic proteins.[8] The fact that complexes of protein with hyaluronate are easier to solubilize than those with sulfated glycosaminoglycans indicates that the interaction depends primarily on the charge density which is probably stronger with the sulfate than with the carboxyl groups.

Precipitation formation of sulfated glycosaminoglycans with lectins, carbohydrate binding proteins, was reported.[291,292] Buonassisi and Colburn[291] showed that acidic pH was optimum for the precipitation, and that high concentration of salt inhibited the precipitation, suggesting that the nature of the interaction was electrostatic attraction.

TABLE 8

Polypeptide-Glycosaminoglycan Interactions: Stoichiometries for Maximum Interaction and Melting Temperatures

	Glycosaminoglycan[a]							
Polypeptide[a]	HA	KS2	C4S	HS	C6S	KS1	DS	HEP
PLA ratio[b]	1:1	1.4:1	2:1	1:1	2:1	1.2:1	1.4:1	34.3:1
conformation[c]	α	α	α	α	α	α	α	α
T_m[d]	35.0	46.0	54.5	65.0	76.0	> 90	> 90	> 90
PLL ratio	1:1	1.4:1	1:1	2:1	1:1	1.2:1	1.4:1	2.3:1
conformation[c]	R	R	R	R	R	R	R	R
T_m[d]	—	—	23.0	—	47.0	—	72.0	> 90
PLO ratio								2.3:1
conformation[c]								α
T_m[d]								56.0

[a] PLA, poly-L-arginine; PLL, poly-L-lysine; PLO, poly-L-ornithine; HA, hyaluronic acid; KS2, keratan sulfate-2; C4S, chondroitin 4-sulfate; HS, heparan sulfate; C6S, chondroitin 6-sulfate; KS1, keratan sulfate-1; DS, dermatan sulfate; HEP, heparin.

[b] Ratio of amino acid to disaccharide residues at maximum interaction.

[c] Induced conformation: α, α-helix; R, random coil.

[d] T_m, melting temperature, in degrees Celsius, for the α-helical effect.

(From Blackwell, J., Schodt, K. P., and Gelman, R. A., *Fed. Proc.*, 36, 98, 1977. With permission.)

As it appeared that more than one binding site was present in the glycosaminoglycans, the authors suggested that the biopolymers might be branched. The precipitation formation reaction was used to isolate various sulfate glycosaminoglycans. Morris and Chan[292] also investigated formation of an insoluble complex between concanavalin A and chondroitin sulfate, hyaluronic acid, and heparin. No precipitation was noted for any glycosaminoglycan in physiological salt solution (about 0.15 *M* NaCl) unless the pH was below 4.5. They characterized the interaction as a reversible nonspecific electrostatic interaction observed only at low pH and ionic strength.

The techniques that have been commonly used for the detection of binding between polysaccharide and protein are electrophoresis, sedimentation, and chromatography in which both components affect each other's transport rate. The existence of a covalent linkage of glycosaminoglycan and proteins in proteoglycan indicates that the interaction between these two macromolecules may not necessarily be electrostatic. The subject — particularly the types of interactions — has been discussed in an excellent recent article by Comper and Laurent.[5]

It is well documented that polyanions, especially heparin, stimulate the nuclear and chromatin transcription process.[293,294] This phenomenon was attributed to the interaction between polyanions and histones, which removes DNA template restrictions and allows RNA synthesis. Complex formation between chondroitin sulfate and histone f_1 fraction on acrylamide gel was reported by Laurence and Higgison.[295] de Pomerai and co-workers[296] demonstrated that heparin affects RNA polymerase activity in a way similar to ammonium sulfate. They suggested that, because of heparin's similar charge property to DNA, heparin would bind to some of the proteins it displaced from chromatin. Binding of heparin to different histone fractions was shown by polyacrylamide gel electrophoresis;[297] in low concentration of heparin, it binds preferentially to histone f_{2a} and f_{2b} fractions. In parallel with this result, Berlowitz et al.[298] showed that the arginine-rich fractions f_{2a1} and f_3 were the first histone fractions to bind to polystyrene sulfonate, whereas the lysine-rich fraction f_1 was the last. Destabilization of chromatin

by various polyanions including heparin was reported by Ansevin et al.,[299] who suggested that the chief factors controlling the dissociation of histones from DNA in chromatin by polyanions are the charge density of the polyanions and steric hindrance in the polyanions that prevents the close approach of charged areas to the nucleoprotein surface. Demidenko and Tsvetkova[300] investigated the effect of heparin on protein-fractions and DNA of isolated hepatocyte nuclei from Wistar rats. They reported that 95% of histone is blocked by exposure of the nuclei to 0.05% heparin, which was related to release of DNA from the nuclei.

The unique biological effects of heparin — its anticoagulant and lipid clearing action — have promoted studies on its interaction with proteins that are involved in biological processes. It has been proposed that heparin induces a conformational change in antithrombin III after its specific binding to heparin.[301,302] Villanueva and Danishefsky[303] demonstrated spectroscopically a conformational change of antithrombin III, a decrease in β-structure with a compensatory increase in random coil of the molecule. However, it has also been reported that heparin binds tightly to thrombin,[304-306] making the mechanism of the action of heparin on the thrombin-antithrombin reaction more complicated.

Electrostatic interaction between sulfated glycosaminoglycans and plasma lipoproteins were demonstrated by the use of gel chromatography at pH 7.4; both very low density lipoprotein (VLDL) and low density lipoprotein (LDL) were bound to agarose gels substituted with sulfated glycosaminoglycans in very low salt concentration, whereas no detectable binding occurred in high salt concentration.[307] A similar result was shown for Lp(a) lipoproteins, but binding in the presence of $CaCl_2$ was reported to be stronger than that in NaCl of the same ionic strength.[308] Nakashima and coworkers[309] isolated various species of plasma glycosaminoglycans. Their fluorescence studies on the mixtures of the polymers and lipoproteins suggested that sulfate-rich glycosaminoglycans caused a change in lipoprotein conformation associated with either an increased volume or increased microscopic viscosity of the hydrocarbon region of the lipoproteins.

Ionic interaction between heparin and lipoprotein were shown by Olivecrona et al.,[310] and the interaction properties were reviewed briefly.[311] L-Iduronic acid-containing glycosaminoglycans — heparin, heparan sulfate, and dermatan sulfate — were effective for the enzyme binding, whereas chondroitin sulfate showed only small binding capacity.[311] (It is interesting to note that dermatan sulfate also exhibits higher affinity for metal ions and H^+ than does chondroitin sulfate.) The possibility of two different binding sites of heparin for antithrombin and lipoprotein lipase was demonstrated by Bengtsson et al.[312]

Cartilage proteoglycans are known to form aggregates with each other in the tissue; the phenomenon has been explained as resulting from interaction between proteoglycan monomers with a single hyaluronate chain.[313-316] Studies[317] with hyaluronate oligosaccharides of varying lengths have demonstrated that the protein in the proteoglycan contains a globular part that binds specifically to a decasaccharide unit in the hyaluronate chains. The binding is stable at high ionic strength, but unstable at high concentration of guanidine hydrochloride. The nature of this binding remains unclear. Interestingly, other polyions such as alginate, dextran sulfate, and DNA do not interact with proteoglycan monomers.

Collagen

Collagen is closely associated with glycosaminoglycans and proteoglycans in the supporting tissue of a wide range of animal species. Even though Katzman and Jeanloz[318] and Becquet et al.[319] observed absence or very low concentration of acidic polysac-

charides in the collagenous mesoglea of two species of coelenterates and questioned whether there was an absolute biological requirement for acidic polysaccharides in the formation and stabilization of collagenous structures, many in vitro investigations have shown interaction between collagen and glycosaminoglycans and noted its regulatory effect on collagen fibril formation.

Jackson[320] showed that chondroitin sulfate, and possibly some other glycosaminoglycans, plays a part in the stabilization of tendon, suggesting 25% of the stabilizing linkages are between collagen and chondroitin sulfate consisting of 40% saltlike links and 60% hydrogen bonds. Mathews[321] studied the interactions of a solubilized collagen with various glycosaminoglycans and observed the reversible formation of complexes of an electrostatic nature between the components. The extent and nature of the complex formation depend on the type of polysaccharides, their molecular weight, and the ionic strength of the medium. A similar complex of ionic character was also shown to form between collagen and proteoglycans by Stevens and co-workers.[322,323]

Chromatographic study of chondroitin 4-sulfate and chondroitin 4-sulfate proteoglycan on a collagen gel by Öbrink and Wasteson[324] indicated that the electrostatic interaction between the macromolecules increased with decreasing pH and ionic strength. Isoelectric focusing analysis of modified proteoglycans and tropocollagen by Podrazky et al.[325] demonstrated that the sulfate group of glycosaminoglycans was largely responsible for interaction with the guanidino groups of arginine and ε-amino groups of lysine of the collagen.

Lowther and Natarajan[326] reported that unaggregated chrondoitin sulfate proteoglycan inhibited collagen fibril formation in vitro for several hours, whereas aggregated proteoglycans containing glycoprotein had no effect. Influence of glycosaminoglycans on collagen fibril formation was reported by Öbrink[327,328] and Németh-Csóka.[245] Light scattering measurement by the former author[327] at physiological ionic conditions showed that hyaluronic acid and keratan sulfate did not bind significantly to lathyritic collagen, whereas chondroitin 4-sulfate, dermatan sulfate, heparan sulfates, heparin, and preoteoglycans of chondroitin sulfate and dermatan sulfate formed complexes of an electrostatic nature with collagen. He also reported that L-iduronic acid-containing polysaccharides interact more strongly than do polysaccharides containing only D-glucuronic acids. Subsequently, he reported[328] the effects of glycosaminoglycans on the two stages of collagen fibril formation, namely, a nucleation phase and a fiber growth phase. The results indicated that chondroitin sulfate and dermatan sulfate accelerated the fiber formation by affecting the nucleation process, but chondroitin sulfate and heparan sulfate fraction and the proteoglycans delayed fiber formation, when added after the nucleation phase, by inhibiting the growth of fiber from the nuclei. Hyaluronic acid, which does not form a complex with collagen, accelerated both processes by steric exclusion, whereas keratan sulfate had no effect or a slightly decelerating effect. Németh-Csóka[245] studied collagen fibril formation with added glycosaminoglycans and various inorganic ions including phosphate and citrate. The results were similar to those of Öbrink,[328] which are shown in Figure 38.

All glycosaminoglycans were found to accelerate fibril formation when the ionic strength was 0.5. In the absence of glycosaminoglycans, sulfate, phosphate, and citrate ions delayed fibril formation, NO_3^- and SO_3^{2-} had no effect, and SON^-, Cl^-, Fl^-, and CO_2^{2-} enhanced the process. Németh-Csóka[245] suggested that the glycosaminoglycans in connective tissue ground substance in fibrillogenesis might regulate the ion binding and, through this, the tendency of aggregation of the collagen molecules. Németh-Csóka and co-workers[256] demonstrated that desulfated heparin enhanced fibril formation.

Electron microscopic examination of gels formed in solutions containing tropocollagen and chondroitin sulfate and proteoglycan from tracheal cartilage was described

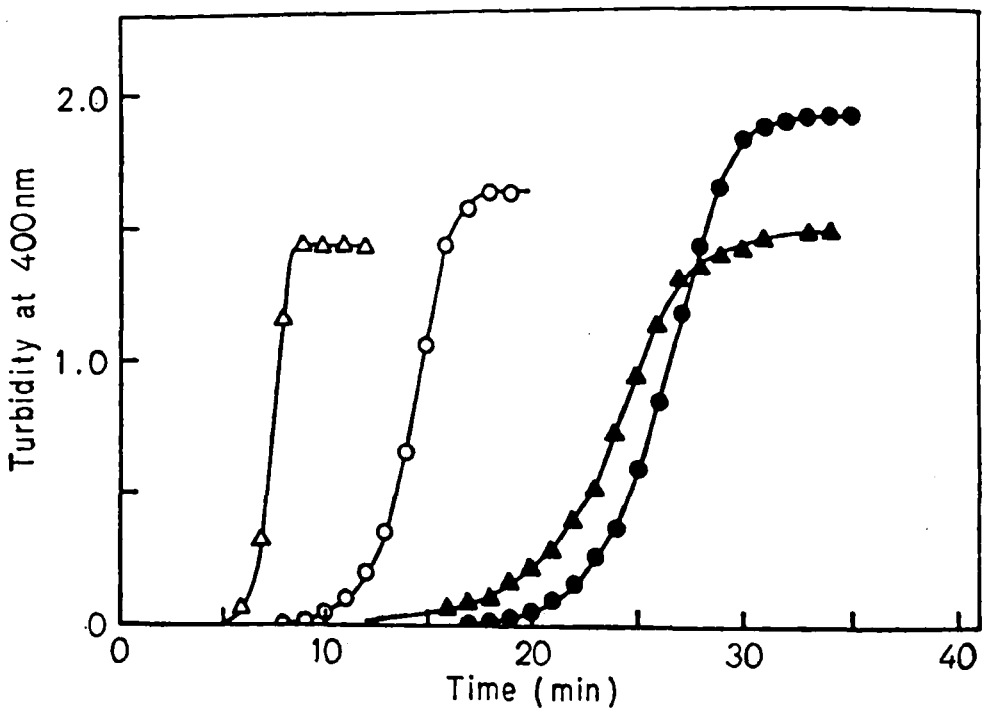


FIGURE 38. Effects of glycosaminoglycans on the gelation rate of 0.8 mg/mℓ collagen. (•) Collagen alone. (Δ) Collagen + 0.5 mg/mℓ dermatan sulfate. (o) Collagen + 0.5 mg/mℓ chondroitin sulfate. (▲) Collagen + 0.1 mg/mℓ chondroitin sulfate proteoglycan. (From Öbrink, B., *Eur. J. Biochem.*, 34, 129, 1973. With permission.)

by Lazarev.[329] Bychkov et al.[330] reported a study on the structural-mechanical strength and swelling of gels consisting of gelatin and hyaluronate or protein-chondroitin 4-sulfate. High structural-mechanical strength of the gels with a minimum of swelling were shown in zones of charge neutrality between gelatin and the macroanions.

Circular dichroic investigation of the melting of collagen in the presence of various glycosaminoglycans was reported by Gelman and Blackwell.[331,332] They demonstrated the interactions of chondroitin 4-sulfate, dermatan sulfate, hyaluronic acid, and keratan sulfate with collagen, which stabilized the helical structure so that the melting temperature of collagen was increased from 38° to 46°C. Each polysaccharide to collagen ratio to achieve the effect differed; below the limiting ratios, biphasic melting curves with transitions at 38° and 46°C, which were attributed to melting of interacting and noninteracting collagen (Figure 39), were obtained.

Based on disaccharide unit per 100 amino acid residues required to achieve the melting temperature of 46°C, the polysaccharides were ordered in terms of the extent of their interaction with collagen: chondroitin 6-sulfate > dermatan sulfate > hyaluronic acid > keratan sulfate > chondroitin 4-sulfate. This order differs from the order of glycosaminoglycan interaction with cationic homopolypeptides (see previous section) and also from the order of influence on fibril formation.

Steric Exclusion

A glycosaminoglycan molecule, flexible linear chains with a random-coil conformation in solution, occupies a large volume and, therefore, resists penetration of its volume by other molecules to a degree dependent upon their size and shape. Such phenomena of molecular exclusion have been discussed for polymers in general by Tanford,[333] and for glycosaminoglycans by Ogston[334] and Laurent et al.[5,9]

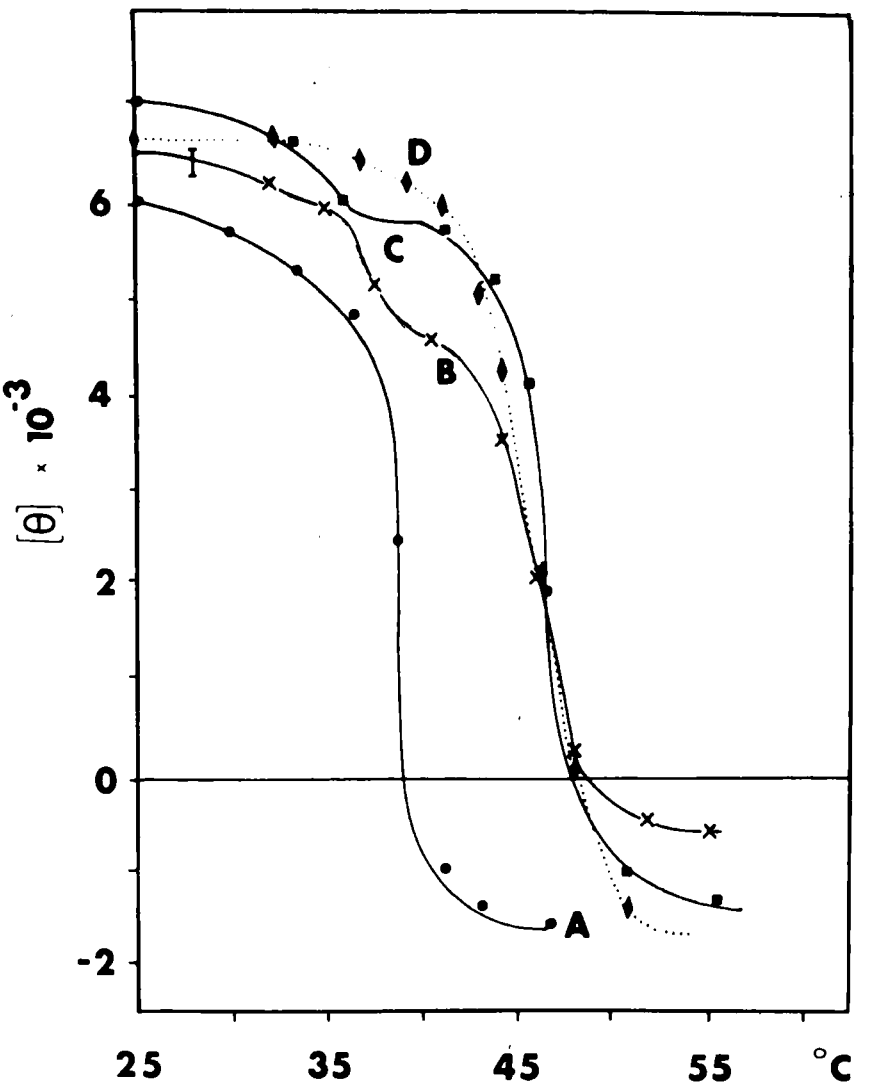


FIGURE 39. Plots of ellipticity at 221 nm, $[\theta]_{221}$, as a function of mixtures of calf skin collagen and hyaluronic acid at various values of number of disaccharide residues (r) per 100 amino acid residues. (A) r = 0, (B) r = 5, (C) r = 10, (D) r = 11. Decrease in $[\theta]_{221}$ with increasing temperature indicates denaturation (melting) of collagen; the increased melting temperature of collagen in the presence of glycosaminoglycans was attributed to stabilization of the collagen triple helical structure. (From Gelman, R. A. and Blackwell, J., *Biochim. Biophys. Acta*, 342, 254, 1974. With permission.)

This excluded volume effect is most pronounced in hyaluronic acid solution because of its large mol wt; 0.2 mg/m*l* hyaluronic acid occupies the entire volume of the solution. The volume occupied by a hyaluronic acid molecule in comparison with other macromolecules is illustrated in Figure 40.

Most sulfated glycosaminoglycans, however, exist in the tissues as components of very large proteoglycan molecules, and thus the proteoglycans and their aggregates can also produce large excluded-volume effects. The physiological significance of such effects and the results of both in vivo and in vitro experiments have been reviewed by Comper and Laurent.[5]

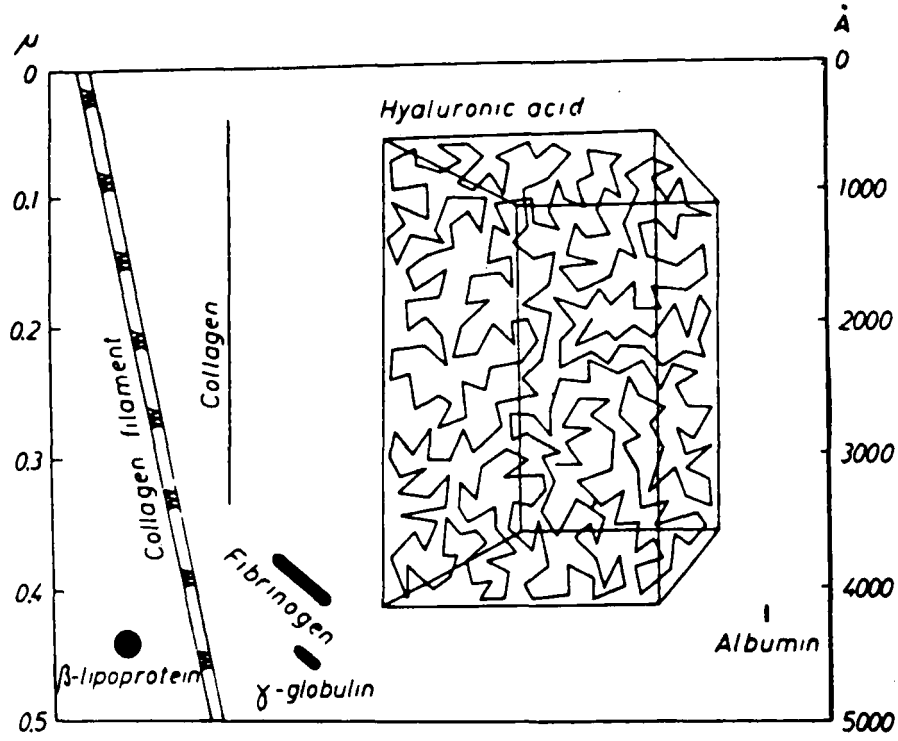


FIGURE 40. Schematic demonstration of the hydrodynamic volume of a hyaluronic acid molecule in comparison with some other macromolecules. (From Laurent, T. C., in *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 2, Balazs, E. A., Ed., Academic Press, London, 1970, 703. With permission. Copyright by Academic Press Inc. (London) Ltd.)

The effects of exclusion have been studied by various techniques including sedimentation and osmometry.[9] Studies by Laurent and co-workers[335,336] and by Preston and Snowden[337] showed that the rate of transport of a particle decreased with increasing particle size and increasing concentration of hyaluronate, following an empirical relationship:[336]

$$s/s^\circ = A \cdot e^{-k \cdot d\sqrt{C_{HA}}} \qquad (11)$$

where s denotes sedimentation velocity of a particle of diameter d in hyaluronate solution of concentration C_{HA}. A and k are constants. Studies by Preston et al.[338] showed that the rotational diffusion of albumin in hyaluronate solutions is essentially unimpeded in conditions in which the translational motion of protein is markedly retarded; this supports the theoretical model of Ogston et al.[339] The linear polymers were less retarded in the hyaluronate network than globular particles of equal hydrodynamic dimensions.[340] The nonspecific and reversible aggregation of red blood cells by addition of hyaluronate and protein-chondroitin 4-sulfate complex was also attributed to the excluded volume effects of the macromolecules.[341]

Shaw[342] and Shaw and Schy[343] measured osmotic pressure of bovine serum albumin (BSA), chondroitin sulfate, hyaluronic acid, and dextran, and their binary mixture solutions. The data were used to calculate number-averaged molecular weight M_n (from first virial coefficients) and excluded volume (from second virial coefficients). Excluded volumes were 8.7 m*l*/g for BSA of M_n 67,000,220 m*l*/g for chondroitin sulfate of M_n 39,000, and 62.1 m*l*/g for hyaluronic acid of M_n 51,000; their data bore a 30% uncertainty.

CONCLUSIONS

It is apparent that the primary structure of glycosaminoglycans is less complicated than that of other classes of biopolymers, such as proteins, nucleic acids, and lipids. Because of the seeming simplicity and apparent dullness of the structure and lack of knowledge about biological function, research on the polysaccharides of living organisms has been consigned to a secondary place, after proteins and nucleic acids. The past few years, because of growing interest in the macromolecular organization of connective tissue components and in their biological relevance, have seen renewed efforts to understand the structure of polysaccharides of animal origin. Referring to polysaccharides in general, Rees[344] said: "They have become interesting molecules to contemplate in relation to the life of the cell. The ugly ducklings have begun to look a little more like swans. In this sense, polysaccharides begin to appear attractive molecules, shapely molecules."

For the last 20 years, the research on glycosaminoglycans has been intensive. In this review, we have emphasized the later work, which is primarily concerned with the structure and interaction of these biopolymers. Studies on hydrodynamic properties have provided a good deal of knowledge regarding size and shape. Although these investigations have revealed that glycosaminoglycans are fairly flexible polymers, very few studies have been directed toward examining the possiblity of a certain degree of order or rigidity of the molecules. Detailed analyses of X-ray crystallographic results for many glycosaminoglycans are still lacking, but the data are enough to indicate some sort of helical structure in the solid state. Spectroscopic investigations have been carried out not only to examine the possibility of such helical structure of these biopolymers in solution, but also to gain information on the fundamental aspects such as origin and assignment of spectral bands, spatial disposition of the chromophores, configuration and conformation of the sugar ring, and interaction properties of the substituent groups. Subtle conformational changes of glycosaminoglycans — which may be biologically important — that may follow a change in pH, solvent, or temperature and interaction with other molecules can be detected only by the combination of spectroscopic techniques, hydrodynamic measurements, and X-ray diffraction studies. Success is noteworthy in the case of hyaluronic acid and is sure to come for other glycosaminoglycans. A major gap still exists in our knowledge of the biological function of these macromolecules at the molecular level, and whether the molecular shape or ionic charge is the major determinant of the function. Comper and Laurent[5] correctly stated in their review article: "The polysaccharide functions seem to be related to their general macromolecular and polyelectrolyte characteristics and hence the physicochemical properties they can confer on the systems and structures that contain them." The task is enormous, but with the growing interest of many investigators in this field, it is hoped that we will be able to share the burden.

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