Critical Reviews in Biochemistry and Molecular Biology, 30(5):387–444 (1995)

Enzymatic Degradation of Glycosaminoglycans

*Steffen Ernst,[1] Robert Langer,[1,2] Charles L. Cooney,[1] and Ram Sasisekharan[2]**

[1]Department of Chemical Engineering and [2]Harvard MIT-Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139

* Corresponding author: Ram Sasisekharan, MIT, 77 Massachusetts Avenue, Building E17-430, Cambridge, MA 02139.

ABSTRACT: Glycosaminoglycans (GAGs) play an intricate role in the extracellular matrix (ECM), not only as soluble components and polyelectrolytes, but also by specific interactions with growth factors and other transient components of the ECM. Modifications of GAG chains, such as isomerization, sulfation, and acetylation, generate the chemical specificity of GAGs. GAGs can be depolymerized enzymatically either by eliminative cleavage with lyases (EC 4.2.2.-) or by hydrolytic cleavage with hydrolases (EC 3.2.1.-). Often, these enzymes are specific for residues in the polysaccharide chain with certain modifications. As such, the enzymes can serve as tools for studying the physiological effect of residue modifications and as models at the molecular level of protein-GAG recognition. This review examines the structure of the substrates, the properties of enzymatic degradation, and the enzyme substrate-interactions at a molecular level. The primary structure of several GAGs is organized *macroscopically* by segregation into alternating blocks of specific sulfation patterns and *microscopically* by formation of oligosaccharide sequences with specific binding functions. Among GAGs, considerable dermatan sulfate, heparin and heparan sulfate show conformational flexibility in solution. They elicit sequence-specific interactions with enzymes that degrade them, as well as with other proteins, however, the effect of conformational flexibility on protein-GAG interactions is not clear. Recent findings have established empirical rules of substrate specificity and elucidated molecular mechanisms of enzyme-substrate interactions for enzymes that degrade GAGs. Here we propose that local formation of polysaccharide secondary structure is determined by the immediate sequence environment within the GAG polymer, and that this secondary structure, in turn, governs the binding and catalytic interactions between proteins and GAGs.

KEY WORDS: hydrolase, kinetics, lyase, mechanism, polysaccharide, structure, sulfation.

ABBREVIATIONS

AT III, antithrombin III; **β-ME,** β-mercaptoethanol; **CD,** circular dichroism; **CZE,** capillary zone electrophoresis; **DTT,** dithiothreitol; **EC,** endothelial cells; **ECM,** extracellular matrix; **EDTA,** ethylenediaminetetraacetic acid; **FAB-MS** fast atom bombardment mass spectroscopy; **FGF,** fibroblast growth factor; **a,bFGF,** acidic; basic fibroblast growth factor; **GAG,** glycosaminoglycan; **glcUA** glucuronic acid; **GPC,** gel permeation chromatography; **HB-EGF,** heparin binding epidermal growth factor; **HBP1,** heparin binding peptide I; **HC II,** heparin cofactor II; **HEPES,** *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; **HPLC,** high-performance liquid chromatography; **HS,** heparan sulfate; **HSPG,** heparan sulfate proteoglycan; **I,** ionic strength; **IAA,** iodoacetic acid; **idoUA,** iduronic acid; **IU,** international units (μmol product formed per minute); $\mathbf{K_d}$, dissociation constant in n*M* or μ*M*; $\mathbf{K_m}$, Michaelis Menten constant in μ*M* disaccharides; **MOPS** 3-(*N*-morpholino)propanesulfonic acid; **NEM,** *N*-ethylmaleimide; **NeuNAc,** neuraminic acid (sialic acid); **PCMB,** *p*-Chloromercuribenzoic acid; **PG,** proteoglycan; **SAX,** strong anion exchange; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **SEM,** scanning electron microscopy; **SMC,** smooth muscle cells; **SOS,** sucrose octasulfatel; **TES,** *N-tris*-(hydroxymethyl)methyl-2-aminoethanesulfonic acid; **TLCK,** *N*-α-*p* tosyl-L-lysine-chloromethyl ketone; **TPCK,** *N*-tosyl-L-phenylalanine-chloromethyl ketone; **UV,** ultraviolet; **ΔU,** 4,5 unsaturated uronic acid.

1040-9238/95/$.50

I. INTRODUCTION

Although detailed understanding of DNA and proteins has provided the foundation for molecular biology, the study of the third major class of biological macromolecules, polysaccharides, only recently has started to gain momentum (Hardingham and Fosang, 1992; Hart, 1992). Polysaccharides are among the most diverse biological macromolecules, consisting of cyclic monomers of varying size and structure, which can be linked through many different bonds and modified extensively. Their biological function involves modulation of extracellular processes and is often mediated by oligosaccharide sequences (Varki, 1993). The function of glycosaminoglycans (GAGs) in the extracellular matrix is an important example of polysaccharide diversity and specificity with high impact in development and disease.

The extracellular matrix (ECM) provides a physical and chemical connection among cells in tissue. In recent years, the importance of the ECM in signal transduction has gained increasing recognition (Folkman and Klagsbrun, 1987; Ingber, 1989; D'Amore, 1990; Sporn and Roberts, 1990; Vlodavsky et al., 1991). Acidic polysaccharides (GAGs), present in the ECM, play a central role in this modulation of cell signals (Jackson et al., 1991; Kjellèn and Lindahl, 1991; Scott, 1992). Modification of functional groups along GAG polymers provides a framework for modulating interactions with cytokines that regulate growth and differentiation, such as fibroblast growth factor (Rapraeger et al., 1991) and vascular endothelial growth factor (Gitay-Goren et al., 1992). GAGs can bind these signal transducers and potentially serve as a reservoir, scavenger, or cofactor for cell signaling (Jackson et al., 1991; Vlodavsky et al., 1991; Varki, 1993; Lindahl et al., 1994). In addition, GAGs are soluble and have hydration functions in the ECM, critical to tissues such as cartilage and cornea (Hardingham and Fosang, 1992; Scott, 1992).

There has been a growing interest in the physiological functions of GAGs. At the tissue level, GAGs and proteoglycans have been related to angiogenesis, glomerular permeability, neuron development, joint function and Alzheimer's disease (Gallagher et al., 1986; Poole, 1986; Celesia, 1991). GAGs also have numerous pharmaceutical applications: as anticoagulant, antithrombotic, and antilipemic agents, as well as in osteoarthrosis (Engelberg, 1991; Hirsch, 1991; Oliviero et al., 1991; Walenga et al., 1991).

GAG degrading enzymes have proven useful in studying structure of GAGs, and mechanisms of GAG activity. These enzymes often are specific for certain sequences in the GAG chain (Desai et al., 1993b), and as such, they are interesting as models to study specific interactions between GAGs and proteins. The substrate specificities of GAG degrading enzymes often have formed the basis for interpretation of composition and structure of GAG chains (Nader et al., 1987; Oeben et al., 1987; Linhardt et al., 1988b; Stuhlsatz et al., 1989; Habuchi et al., 1992).

Lately, the GAG degrading enzymes themselves also have shown a potential for pharmaceutical and diagnostic applications; experiments have been conducted with injection of chondroitinase to relieve intraocular pressure (Sawaguchi et al., 1992) and with heparinase to inhibit neovascularization (Sasisekharan et al., 1994). A clinical heparin assay based on quantitative enzymatic degradation has been approved by the FDA (Baugh et al., 1992; Tejidor et al., 1993). Also, heparinase I has been immobilized in a bioreactor for heparin neutralization after extracorporeal therapies (Langer et al., 1982). *In vivo*, degradation of GAG chains by hydrolases is intricately connected to cell mobility in metastasis, inflammation and angiogenesis (Nakajima et al., 1988; Ishai-Michaeli et al., 1990).

Interestingly, genetic defects in GAG *metabolic* enzymes result in viable offspring with severe clinical manifestations (mucopolysaccharidosis) (Sjøgren et al., 1987; Hopwood, 1989), while to the best of our knowledge there are no deficiencies in GAG *anabolic* enzymes that are not lethal at the embryonic state (Hart, 1992; Varki, 1993). These observations suggest that GAGs typically are involved in multicell processes and are of fundamental importance in development. They also suggest the use of GAG-degrading enzymes, expressed or injected in a tissue, for investigating the effect of eliminating a given GAG without knocking out the gene encoding an enzyme nec-

essary for its synthesis (Varki, 1993). Using this approach, it was demonstrated that heparinase injection randomized the left-right axial orientation (Yost, 1992) and prevented mesoderm formation (Itoh and Sokol, 1994) in *Xenopus* embryos.

This paper reviews the recent literature on enzymatic depolymerization of mammalian GAGs. This group of polysaccharides includes: heparin, heparan sulfate, chondroitin sulfates, dermatan sulfate, keratan sulfate, and hyaluronic acid. Enzymatic degradation of GAGs is examined at two levels:

- Observations of the effect of enzymes on their substrates (optimal conditions, substrate specificity, product profile, and effect of inhibitors) provide empirical rules for how the enzymes work.
- Detailed molecular models for enzyme-substrate interaction provide the basis for understanding mechanisms of catalysis, and eventually can lead to rational approaches to modify the function of the enzymes or designing inhibitors.

These two levels of understanding serve as a theme for this review. The substrates and their structures are presented and analyzed in the context of specific binding to proteins. Then, the literature on GAG degrading enzymes is analyzed, and general rules for enzyme specificity are presented. Finally, this information is combined to conceptualize the structure-function relationship of GAG depolymerization. Throughout the review, physical and chemical mechanisms that control the degradation are emphasized.

II. SUBSTRATES

Enzyme-substrate interaction is a three dimensional event involving the interactions of binding and catalytic domains of the enzyme. To fully appreciate the nature and specificity of enzyme function, it is necessary to understand the structure of both the substrate and the enzyme.

GAGs serve four types of functions in the body: as barriers to diffusion across basement membranes, as sources of mechanical repulsive forces for lubrication and cushioning in joints, as anticoagulant coatings of blood vessels, and finally as reservoirs for specific binding to proteins, presumably to regulate or stabilize their activity (Gospodarowicz and Cheng, 1986; Rosengart et al., 1988; Saksela et al., 1988; Sommer and Rifkin, 1989). The tissue localization of the various GAGs has been reviewed elsewhere (Fransson, 1985; Nieduszynski, 1985; Poole, 1986; Nader and Dietrich, 1989; Kjellèn and Lindahl, 1991).

A. Common Features of GAGs

GAGs are polymers of repeated disaccharide units consisting of an uronic acid (U) and a hexosamine (H)*. Biosynthesis of all GAGs, except hyaluronic acid, is initiated from a core protein. GAGs are *O*-linked to serine through a linkage sequence at its reducing end: [GAG]-GlcUA-β1,3-Gal-β1,4-Gal-β1,4-Xyl-β1,*O*-Ser (Poole, 1986), however, for keratan sulfate alternative linkage regions are possible (Section II.D). Core proteins containing several GAG side chains, from similar or different families, are called proteoglycans (PG). GAGs are synthesized as homopolymers which may subsequently be modified by *N*-deacetylation and *N*-sulfation, eventually followed by C5-epimerization of glucuronic acid (GlcUA) to iduronic acid (IdoUA), and *O*-sulfation (Balduini et al., 1989; Evered and Whelan, 1989; Hascall and Midura, 1989; Lindahl et al., 1989). Table 1 provides an overview of the chemical structure and possible modifications for each of the four families of GAGs. GAGs are among the most negatively charged biopolymers, due to uronic acids in the backbone and the often extensive sulfation.

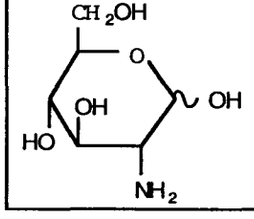
The chemical composition of GAGs from various tissues varies highly. The individual chains in a GAG preparation have different molecular weights and compositions. GAG chains from the same tissue, which are linked to different core proteins may have differences. These differences

* The degree of polymerization, n, is the number of monosaccharide units.

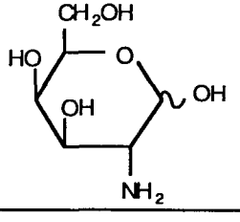
TABLE 1
Repeating Disaccharide Units of GAGs and Their Modifications

	Disaccharide residue [a]	H	U	Modifications
Heparin and heparan sulfate	$H_{2NY,6X,3X}$ -(α1,4) - U_{2X} -(α/β1,4)	Glucosamine	Iduronic acid (I) or glucuronic acid (G)	N-sulfation C5-epimerization O-sulfation
Chondr. sulfates and dermatan sulfate	$H_{2NAc,4X,6X}$ -(β1,4) - U_{2X} -(α/β1,3)	Galactosamine	Iduronic acid (I) or glucuronic acid (G)	C5-epimerization O-sulfation
Keratan sulfate	$H_{2NAc,6X}$ -(β1,3)- Gal_{6X}-(β1,4)	Glucosamine	Galactose	O-sulfation
Hyaluronic acid	H_{2NAc} - (β1,4) - G -(β1,3)	Glucosamine	Glucuronic acid	None

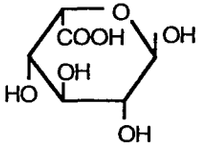
D-Glucosamine:



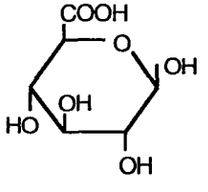
D-Galactosamine:



α-L-Iduronic acid:



β-D-Glucuronic acid:



[a] X = This site is either sulfated or unsubstituted. Y= This site is either sulfated, acetylated, or unsubstituted.

among the individual chains in a GAG preparation are termed 'macroheterogeneity'.

Within a single GAG chain, there can be significant 'microheterogeneity' or compositional differences among residues. Specially modified disaccharides can be segregated into regions which constitute functional sites in the chain. The modification is controlled by biosynthetic enzymes (Balduini et al., 1989; Hascall and Midura, 1989; Lindahl et al., 1989), but it has not yet been clarified how clustering of certain modified residues is controlled.

The structure of GAG polymers can be described at several levels, somewhat analogous to protein structure. The primary GAG structure is defined by the overall composition of particular preparations, as well as the sequence of special functional sites, while the secondary structure is the spatial appearance of a chain, (e.g., repeated helical winding). One could define a tertiary structure of GAGs to describe the tendency of chains to fold on themselves, however, such folding of GAGs has not been described in the literature. Similarly, a quaternary level of GAG structure could be the association of chains into oligomers; although these can be demonstrated experimentally, their physiological significance is still unclear and, for the purposes of this review, higher order interactions among GAG chains or between GAGs and proteins will be broadly classified as *intermolecular associations.*

It is becoming clear that specificity in intermolecular associations is correlated to the primary sequence of residues in the chain. It is not known, however, what role the secondary structure of the GAG chain plays in these interactions. For free GAG chains in solution, the degree of flexibility and secondary structure formation is an area of research.

B. Heparin and Heparan Sulfate

Heparin and heparan sulfate are composed of a glucosamine linked 1,4 at both reducing and non-reducing ends to an uronic acid (Table 1). They are modified by *O*- and *N*-sulfation in addition to isomerization of the uronic acid and are the structurally most diverse glycosaminoglycans.

The macroheterogeneity of heparin and heparan sulfate is highly dependent on the core protein (Poole, 1986). Heparin is found primarily in mast cells and is synthesized with a molecular weight of 50 to 100 kDa (100 to 200 disaccharide residues) (Lindahl et al., 1989). However, during its life cycle the molecular weight is often decreased to an average of 13 kDa (Linhardt et al., 1992b). Heparan sulfate proteoglycans (HSPG) are believed to be present on the cell surface of all human cells. On fibroblast and hepatocyte cell surfaces core proteins have 4 to 6 chains of 14 to 20 kDa each, while in basement membranes as many as 12 heparan sulfate chains varying from 25 to 70 kDa are attached to each protein anchor (Gallagher et al., 1986; Poole, 1986).

1. Primary Structure

The primary structure of heparins and heparan sulfates comprises both an overall chemical composition as well as specific sequences of residues.

Various criteria for definition of heparin and heparan sulfate have been debated in the literature (Comper, 1981; Hopwood, 1989). In the present paper, heparin and heparan sulfates will be considered a family of molecules with a continuous range of compositions and properties. The term heparin represents molecules from that family with more than 0.8 *N*-sulfates and 1.5 to 2 *O*-sulfates per disaccharide residue, while heparan sulfate has one to two sulfates per disaccharide and a significant degree of *N*-acetylation of glucosamines (more than 10%).

The overall content of uronic acids was initially determined by carbazole assay (Bitter and Muir, 1962) or by modification with carbodiimide followed by [^{3}H]borohydride reduction (Taylor et al., 1973), but these were subject to several sources of error (Comper, 1981). Instead, the composition has been determined by chemical and enzymatic degradation, followed by identification and quantitation of the products. Chemical with nitrous acid, which is specific for degradation *N*-sulfated or free amino groups in the hexosamine, may cause reduced yield due to ring-contraction of uronic acid residues or β-elimination of the glucuronic acid (Guo and Conrad, 1989). Enzy-

matic degradation with heparin and heparan sulfate lyases mask the isomer of uronic acid in a cleaved bond because of an unsaturated (Δ4,5) bond formed by the enzyme. Compositions of heparins and heparan sulfates using these chemical and enzymatic degradation methods are given in Table 2.

To validate a compositional analysis it is important to account for the full mass of the substrate and thus, close the mass balance. This often has presented a problem in GAG analysis, and unfortunately few authors include the degree of recovery with their results. For enzymatic degradation, the identified products in some cases can account for up to 90% of the starting material (Linhardt et al., 1992a), whereas the identified products and byproducts recovered from nitrous acid degradation of heparan sulfate (Guo and Conrad, 1989) may constitute only 50% by mass of the starting material (Ernst, unpublished observations). Furthermore, when ^{35}S labeling is used to quantitate degradation products of heparan sulfate (Marcum and Rosenberg, 1989b; Kojima et al., 1992), non-sulfated disaccharide degradation products are not detected, and the overall composition reported will be biased toward high degrees of sulfation.

As shown in Table 2, the heparin preparations are relatively constant in their composition with 85 to 95% *N*-, 6-*O*- and 2-*O*-sulfation, and 90% iduronic acid content (Linhardt et al., 1988b; Casu, 1989). Only sea animal heparins have a lower degree of sulfation and a higher glucuronic acid content (Pejler et al., 1987b). Heparan sulfate chains from mammals are more diverse and especially the degree of *O*-sulfation varies from 0.04 to around 1.4 per disaccharide residue. Also, the iduronic acid content varies from 25 to 80% of the uronic acids (Comper, 1981; Gallagher and Walker, 1985; Nader et al., 1987; Guo and Conrad, 1989; Marcum and Rosenberg, 1989b; Habuchi et al., 1992; Kojima et al., 1992).

The sequence of modified disaccharides within the chain is an important element of the primary structure of heparins and heparan sulfates. It has become apparent that some GAGs have a high information content embedded in this sequence. Vegradative lyases that are specific for certain patterns of sulfation and isomerization (Section III.A) are used to cleave susceptible residues specifically, thus elucidating the chain sequence.

An *overall* organization of the heparan sulfate chain sequence is found for human fibroblasts and other animal cells (Gallagher et al., 1986; Nader et al., 1987; de Agostini et al., 1990; Gallagher et al., 1992a). The sequence of residues has been mapped by isolating degradation products containing the linkage to the core protein. The 45 kDa heparan sulfate from human skin fibroblasts, is divided in "NS" regions of 2 to 9 disaccharides (1 to 4 kDa) with heavy sulfation and a mixture of IdoUA and GlcUA, and "NAc" regions of 20 to 25 disaccharides (9-11 kDa) with light sulfation and no IdoUA (Lindblom et al., 1991; Turnbull and Gallagher, 1991b; Turnbull and Gallagher, 1991a; Gallagher et al., 1992b; Gallagher et al., 1992a). The core protein is linked to an NAc region, and the first linkage susceptible to heparinase I (containing I_{2S}) is found either in the first NS region 16 disaccharides from the linkage, or in the second NS region around residue number 35 (Figure 1). Similar clustering of certain functional groups has been found for dermatan sulfate and keratan sulfate (Sections II.C and II.D).

Two functional sequences in the chain have been discovered that involve specific modification patterns:

For heparin, the anticoagulant activity of the molecule is directly correlated to the presence of a region that binds to antithrombin III (AT III). This region is a pentasaccharide ($\mathbf{H}_{NS/Ac,6S}$-**G**-$\mathbf{H}_{NS,(6S),3S}$-$\mathbf{I}_{2S}$-$\mathbf{H}_{NS,6S}$) (Lindahl et al., 1984; Atha et al., 1985; Linhardt et al., 1988b; Marcum and Rosenberg, 1989a; Loganathan et al., 1990; Yamada et al., 1993), but it needs to be present in a molecule of at least 12 to 16 saccharides to have full anticoagulant activity (Marcum and Rosenberg, 1989a). There is room for slight variations in the sequence; the first hexosamine can be either *N*-sulfated or *N*-acetylated, or the second hexosamine can be without 6-sulfation and still have anticoagulant activity (Lindahl et al., 1984; Atha et al., 1985; Yamada et al., 1993). Commercial heparin chains contain about 4 to 7% by weight of the pentasaccharide, corresponding to an average of one domain per 2 to 3 heparin chains (14 kDa) (Linhardt et al., 1988b; Yamada

TABLE 2
The Overall Molar Composition of Heparins and Heparan Sulfates

Content[a] of	% 3-O S	% 2-O S	% 6-O S	% N S	% GlcUA	Recovery	Method
Heparin							
Porcine intestinal[b]	3	78	93		13	79%[c]	Chemical [1,d]
Porcine intestinal (eight commercial products)	3	86	97	97	~11	86%[e]	Enzymatic [2]
Bovine lung[b]	5	84	92		13	90%[c]	Chemical [1]
Bovine lung (two commercial products)	1	92	97	99	~6	112%[e]	Enzymatic [2]
Clam	1	57	56		34	101%[c]	Chemical [3]
HS							
Porcine aorta	—	8	12	29		95%[c]	Enzymatic [4]
Porcine intestine	4.3	61	78	87	26	50%[e] 90%[c]	Chemical [5]
Rabbit aorta (EC culture)	—	18	37	68	75		Enzymatic [6]
Mouse tumor	—	0.8	3	68			Enzymatic [4]
Mouse microvasculature (EC culture)	—	76	52		23		Chemical [7]
Bovine aorta (EC culture)	1.5	76	32	~40	21	100 %[c]	Chemical [8,9]
Bovine lung	—	81	27		12	12%[e]	
Eleven different cell lines mouse, rat, and human fibroblast, liver, and tumor		2-O and 6-O S: 20-75		40–50			Chemical [10]

a The content of a given compound is based on degradation of the polymers and quantitation of the products. From the reported product distributions, we have calculated the content as moles divided by the total moles of disaccharide detected.
b This value is for a fraction with high affinity for AT III.
c Recovery based on moles of identified products divided by the total moles of detected products. Even if this value is high, large proportions of the substrate. can still be undetected if they appear as long oligosaccharides in the product mix.
d [1]Casu, 1989; [2]Linhardt, et al.,1988b; [3]Pejler et al., 1987b; [4]Habuchi et al., 1992; [5]Guo and Conrad, 1989.; [6]Nader et al., 1987; [7]Kojima et al., 1992; [8]Marcum and Rosenberg, 1989b; [9]Comper, 1981; [10]Gallagher and Walker, 1985.
e Recovery based on mass of detected products divided by initial mass of substrates.

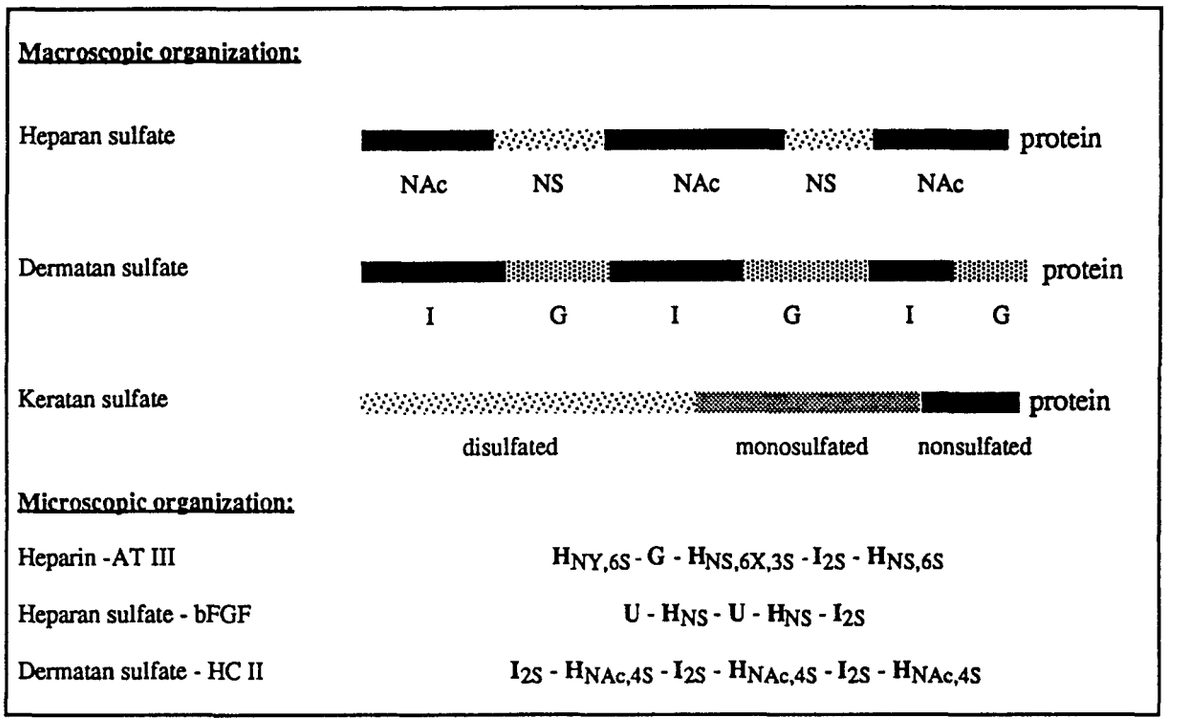


FIGURE 1. Organization of the primary structure of GAGs. Heparan sulfate and dermatan sulfate contain regions with iduronic acid and high degree of sulfation ("NS" or "I") and regions with glucuronic acid and lower degree of sulfation ("NAc" or "G"). In keratan sulfate, a few residues closest to the core protein are non-sulfated followed by a region of monosulfated disaccharides and a region of disulfated disaccharides. Heparin, heparan sulfate and dermatan sufate contain sequences with specific affinity for AT III, bFGF, and HCII, respectively (X = This site is either sulfated or unsubstituted. Y = This site is either sulfated, acetylated, or unsubstituted).

et al., 1993). One third of the heparin molecules have high anticoagulant potential, and most of these contain one such pentasaccharide (Lane and Lindahl, 1989), hence the AT III binding pentasaccharides seem to be evenly distributed. Recently, it has been shown that heparan sulfate also has anticoagulant activity; however, the activity varies considerably with the source of heparan sulfate. Subfractions with high content of the disaccharide **G-$H_{NS,(6S),3S}$** are active (Pejler et al., 1987a; Marcum and Rosenberg, 1989b; Kojima et al., 1992), indicating that the binding sequence is specific and similar to that of heparin.

Also, bFGF binds specifically to a subset of heparin and heparan sulfate molecules (Habuchi et al., 1992; Turnbull et al., 1992; Guimond et al., 1993; Isihara et al., 1993; Maccarana et al., 1993; Tyrrell et al., 1993). The minimal sequence required for binding to bFGF is a pentasaccharide (**U-H_{NS}-U-H_{NS}-I_{2S}**). Additional 2-*O*-sulfation of uronic acids in the pentasaccharide does not affect binding (Maccarana et al., 1993), while 6-*O*-sulfation of hexosamines may (Habuchi et al., 1992; Turnbull et al., 1992) or may not (Maccarana et al., 1993) impair bFGF binding. Cell-surface HSPG serves as a necessary co-receptor for bFGF binding to bFGF-receptor and stimulation of intracellular signaling (Rapraeger, 1993). Although penta and hexasaccharides can support bFGF binding and competitive inhibition of bFGF mediated cell proliferation, deca or dodecasaccharides are necessary to support mitogenic activity of bFGF to cells without HSPG receptors (Guimond et al., 1993; Isihara et al., 1993). Furthermore, these additional residues must be 6-*O*-sulfated and 2-*O*-sulfated to promote receptor signaling by bFGF (Guimond et al., 1993). These studies suggest that heparan sulfate from

HSPG receptors contains a pentasaccharide site with I_{2S} at the reducing end, which is responsible for interaction with bFGF, and an adjacent site of 4 to 6 saccharides with I_{2S} and H_{6S} responsible for modulating the mitogenic signal. This modulation has been proposed to be mediated either directly by binding to the high affinity receptor (Kan et al., 1993), or indirectly by induction of structural changes in bFGF (Prestrelski et al., 1992) or dimerization of both a/b FGF (Ornitz et al., 1992; Mascarelli et al., 1993) that allow FGF to activate the high-affinity receptor kinase.

The rare 2-sulfated glucuronic acid has been shown to be concentrated in the nuclear pool of heparan sulfate in hepatocytes. Heparan sulfate is transported to the nucleus by a non-lysosomal pathway. Because most of the internalized heparan sulfate is metabolized in the lysosomes, the presence of G_{2S} is a likely nuclear localization signal (Fedarko and Conrad, 1986; Isihara et al., 1986; Isihara et al., 1987; Gallagher et al., 1992b).

Literature on the primary structure of heparin and heparan sulfate has shown that these are highly variable in their overall composition, and that the distinction between heparin and heparan sulfate is somewhat arbitrary (Comper, 1981; Hopwood, 1989). Specific sequences of modified disaccharides were found to be involved in AT III and bFGF binding as well as in cellular localization (Lindahl et al., 1984; Isihara et al., 1986; Maccarana et al., 1993). Similarly, sequences of heparan sulfate specific for binding to lipoprotein lipase (Parthasarathy et al., 1994) and hepatocyte growth factor (Lyon et al., 1994) are beginning to emerge.

2. Secondary Structure

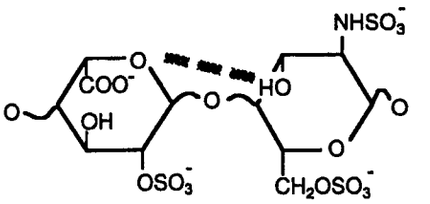
The secondary structure of heparin and heparan sulfate determines how the functional groups of specific sequences are oriented, and provide a basis for understanding how they interact with the binding sites of AT III and bFGF as well as with the active sites of the GAG degrading enzymes. Early studies of helix formation in crystalline fibers, reviewed by Rees et al. (1982), and Nieduszynski (1985) led to possible structures of heparin and heparan sulfate in solid state and in solution. Recently, two-dimensional NMR and molecular modeling have focused on the conformational flexibility of iduronic acid monomer residues (Ferro et al., 1990; Mulloy et al., 1993).

X-ray fiber diffraction analysis of heparin has indicated that it can attain two different 2_1 helices (twofold symmetry with 1 turn per unit cell) with a rise of 0.82 to 0.84 and 0.87 nm/disaccharide respectively, depending on humidity (Atkins and Nieduszynski, 1976; Rees et al., 1982; Nieduszynski, 1985). The sodium salt of heparan sulfate is found in an extended 2_1 helix with axial rise of 0.93 nm/disaccharide, while the calcium salt yields a diffraction pattern with a rise of 0.84 nm/disaccharide, similar to that of heparin (Rees et al., 1982; Nieduszynski, 1985). From model-building of the heparan sulfate extended helix, hydrogen bonding between the acetamido oxygen of an *N*-acetylated hexosamine and the C3 hydroxyl group of the glucuronic acid on the reducing side appears very likely. In models of both helices, hydrogen bonding between the ring oxygen of uronic acid and the C3 hydroxyl of hexosamine seem to stabilize the helix (Figure 2) (Rees et al., 1982). Sulfation at the C3 hydroxyl, which has been observed in the AT III binding sequence, would interfere with this hydrogen bond, and we suggest this may contribute to the specific binding properties of the AT III binding sequence.

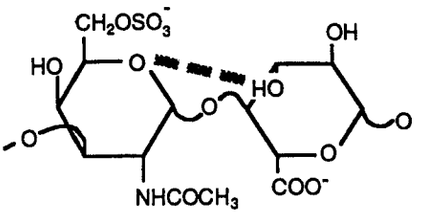
Often, simulations of protein-heparin interactions in solution (Section II.G) are based on modeling heparin as a homopolymer $(\mathbf{I}_{2S}\text{-}\mathbf{H}_{NS,6S})_m$ in the helix form found from diffraction. It is most likely, however, that heparin and heparan sulfates in solution are dynamic entities that populate the various thermodynamically allowed conformations, and that the results of fiber diffraction only can serve to indicate *possible* conformations for dissolved chains. To refine the modeling of heparin, especially its specific sequences, it is important to establish how variations in the disaccharides contribute to the stability of a secondary structure.

For glucuronic acid, like glucose, the $^4C_1$ pyranose pucker conformation is highly favored over the $^1C_4$ pucker (Stoddart, 1971; French and Brady, 1990). However, when glucuronic acid has been epimerized to iduronic acid during heparin biosynthesis, the carboxylate group will be

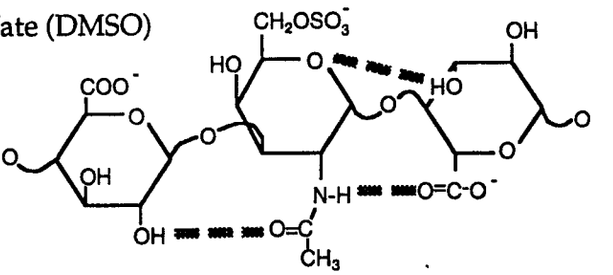
Heparin



Chondroitin 6-sulfate



Chondroitin 4-sulfate (DMSO)



Hyaluronic acid (DMSO)

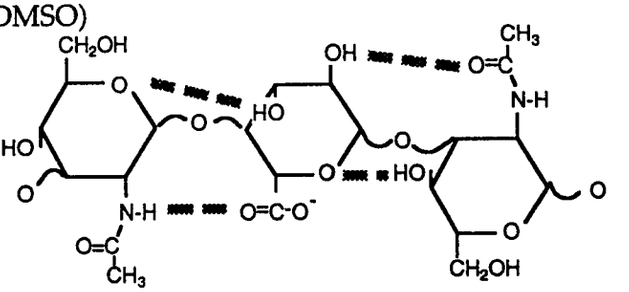


FIGURE 2. Hydrogen bonds (▪▪▪ ▪▪▪ ▪▪▪) between adjacent residues in GAG helices. See text for details.

axial for the $^4C_1$ pucker, and the two conformers are almost equienergetic. Usually, rotation around the glycosidic dihedrals (ϕ = H1-C1-O4-C4 and ψ = C1-O4-C4-H4 for a 1-4 glycosidic bond) is considered the only possible conformational flexibility of polysaccharides (French and Brady, 1990), but in the case of IdoUA, variation in sugar puckers (i.e., rotation around the endocyclic bonds) must also be taken into account. In the crystal models, the $^1C_4$ (chair)* conformer was used for IdoUA, but energy minimization of an IdoUA monosaccharide showed that ^2T_O (twist boat) and $^4C_1$ were equienergetic with $^1C_4$ within 0.8 kcal/mol (Ragazzi et al., 1986). The relative populations of these conformers were estimated by calculating NMR vicinal coupling constants (J val-

* Nomenclature of sugar pucker conformations is from Haasnoot (1992).

ues) for each, and comparing them to the experimental values obtained for various solutions of oligosaccharides that were assumed to represent a weighted average of the contributions from these three iduronic acid conformers (Casu et al., 1988; Ferro et al., 1990) (Table 3). There is a remarkable difference between the conformation of internal and terminal iduronic acids, the latter being shifted toward $^4C_1$ conformation. Also, neighboring sulfates were important for iduronic acid conformation: 3-O sulfation of the hexosamine to the non-reducing side, which is typical for the AT III binding site, shifts the population towards the ^2T_O form, *N*-sulfation on the reducing side in a tetramer causes increased $^1C_4$ population, and finally, 6-sulfation on the reducing side of the iduronic acid has no influence on the conformation (Ferro et al., 1990). As of yet, no force field calculations have been published that attempts to model the effects of neighboring residues.

These results show that the primary sequence of heparin and heparan sulfate may determine the conformation of the iduronic acid residues. The conformation is, in turn, essential for how the charged functional groups are oriented with respect to the helical axis of oligosaccharides (Ragazzi et al., 1990; Mulloy et al., 1993; Venkataraman et al., 1994). For these reasons, it is necessary to address the issue of monosaccharide conformation when attempting to model protein-heparin interactions.

3. Intermolecular Associations

In addition to the secondary structure of each chain heparan sulfate the self-association is important for its function. Certain fractions of heparan sulfate chains self-associate with K_D values around 220 μM at NaCl concentrations above 0.15 M (Fransson, 1985; Nieduszynski, 1985; Gallagher et al., 1986; Scott, 1992). Aggregating heparan sulfates appear to specifically associate with heparan sulfates of the same charge density, which may be a means of cell-cell recognition (Fransson et al., 1981). Thus, specificity in GAG-GAG recognition may be analogous to the sequence specificity that has been demonstrated for protein-GAG interactions.

In summary, the primary structure of heparin and heparan sulfate contain information that determines the specificity of the interaction of these molecules with proteins and with other GAGs.

C. Chondroitin and Dermatan Sulfates

Chondroitin and dermatan sulfates arise from the same homogeneous precursor, chondroitin, with the repeated disaccharide unit:

$$- Gal_{NAc} — (\beta 1,4) — GlcUA\ (\beta 1,3) -$$

Subsequent modification may involve C5-epimerization of glucuronic acid to iduronic acid, and/or *O*-sulfation at C2 of uronic acids and C4 or C6 of galactosamine (Table 1), resulting in a complex primary structure from which dermatan and chondroitin sulfate are derived, much like for heparin and heparan sulfate (Conrad, 1989).

Dermatan sulfate contains iduronic acid to a varying degree (Table 4), whereas all the uronic acids of chondroitin sulfate are glucuronic acid (Conrad, 1989). Dermatan sulfate occurs predominantly in small proteoglycans with 2 to 8 side

TABLE 3
Population of the Three Conformers for Iduronic Acid in Various Sequence Environments (Ferro et al., 1990)

Sequence	Typical for	$^1C_4$	^2T_O	$^4C_1$
... - H_{NS}-I_X-H_{NS} - ...	Heparin	55–60%	35–40%	0
... - $H_{NS,3S}$-I_S-H_{NS} - ...	AT III binding sequence	30–35%	65–70%	0
... - H_{NAc}-I-H_{NAc} - ...	HS	47%	53%	0
I_S-H_{NS} - ...	Nonreducing terminus	80–95%	0	5–22%
I -$H_{NS/NAc}$ - ...	Nonreducing terminus	19–45%	14–26%	29–67%

TABLE 4
Compositional Analyses of Dermatan and Chondroitin Sulfates

Tissue (MW)	% 2-O S	% 4-O S	% 6-O S	IdoUA%	Method	Ref.
Dermatan sulfate						
Porcine skin	5–10[a]	80–83	11–17	80	[b]	Maimone and Tollefsen, 1990
Bovine intestine (30–50 kDa)	10—12	90–96	9–12	84–91	[b]	Linhardt et al., 1991
Porcine intestine (35 kDa)	10–11	87–88	15–21	81–93	[b]	Linhardt et al., 1991
Skin, sclera, cartilage (15–24 kDa)				35–85	[b] or [c]	Poole, 1986
Cornea, glia, embryonic tissue (40–56 kDa)				7–12	[b] or [c]	Poole, 1986
Chondroitin 4-sulfate						
		>79	20	—	[b]	Michelacci and Dietrich, 1975
Chondroitin 6-sulfate		2.5	>80	—	[b]	Michelacci and Dietrich, 1975

[a] The 2-*O* sulfation has been observed both at iduronic and at glucuronic acids of the dermatan sulfate.
[b] Composition was determined from degradation of GAG followed by identification and quantitation of the products.
[c] The extent of a chemical or enzymatic degradation reaction was followed, and the composition was deduced based on the specificity of the acids or enzymes used.

chains of 15 to 55 kDa; chondroitin sulfate is found more often in large aggregating proteoglycans with 20 to 100 GAG chains of 15 to 70 kDa (Poole, 1986).

1. Primary Structure

The overall composition of chondroitin and dermatan sulfates have been determined for tissue extracts and for purified proteoglycans (Table 4). Dermatan sulfates are predominantly 4-sulfated, and the iduronic acid content varies from 7 to 93%. Some of the reported low values of iduronic acid content (Poole, 1986) are based on overall susceptibility to enzymes or periodate oxidation, without identifying the products and therefore may be biased. Among the chondroitin sulfates, copolymers with both 4-*O*- and 6-*O*-sulfation in the same chain are common (Rees et al., 1982), and individual residues that are sulfated at both 4 and 6 position also have been found (Linhardt et al., 1991).

In dermatan sulfate and chondroitin sulfate, two levels of organization of the sequence of residues have been observed (Figure 1). Clustering of residues in dermatan sulfate was evidenced by a new mapping technique, using ^{125}I-labeling of serine from the linkage region, which indicated that the GlcUA residues are concentrated around positions 1-3, 8-9 and 25 from the linkage region at the reducing end (Fransson et al., 1990). Also, the 6-*O* sulfation of chondroitin sulfate seems to be clustered near one or both termina of the chain, depending on the tissue source of chondroitin sulfate (Cheng et al., 1992).

Specific functional sequences are present, as indicated by the finding that, among hexasaccharides, only those containing 2-sulfation have heparin cofactor II (HC II) activity (Maimone and Tollefsen, 1990). Hexasaccharides containing three 2-sulfated iduronic acids are the most active and constitute 2% of all hexasaccharides, far more than would be expected if this uncommon residue was randomly distributed (Maimone and Tollefsen, 1990; Tollefsen, 1992). Thus, a relatively rare residue has an important physiological function. This is analogous to the function of 3-sulfation of glucosamine in the AT III binding site of heparin. Thrombomodulin, a cell surface proteoglycan which alters thrombin activity and is a cofactor for activation of protein C, has one chondroitin sulfate side chain, which is characterized by a terminal tetrasulfated trisaccharide sequence ($\mathbf{H}_{NAc,4S,6S}$-**G**-$\mathbf{H}_{NAc,4S,6S}$) (Bourin et al., 1990; Bourin and Lindahl, 1993). The chondroitin sulfate chain is necessary, but not sufficient for full inhibition of thrombin; however, the role of the unusual trisaccharide at the non-reducing end has not yet been determined.

2. Secondary Structure

There is a pronounced difference between dermatan sulfate and chondroitin sulfate with respect to secondary structures. Dermatan sulfate seems to have a flexible conformation in solution, (Caso et al., 1988; Venkatareman et al., 1994), on the other hand chondroitin sulfate forms defined secondary structures.

In solid state, however, helices of various periodicities have been shown by diffraction patterns of both dermatan and chondroitin sulfate (Table 5). Fibers of chondroitin 4-sulfate attain a twofold helix when crystallized with Ca^{2+} and a three-fold helix with Na^+ (Cael et al., 1978; Mitra et al., 1983). Similarly, the helix of chondroitin 6-sulfate is dependent on the counterion, while all three helices of dermatan sulfate have been obtained with Na^+ (Millane et al., 1983; Nieduszynski, 1985). These solid state structures are stabilized by hydrogen bonding and chelation of calcium ions. The most frequently observed hydrogen bond is formed by the ring oxygen of galactosamine to the 3-hydroxyl of glucuronic acid, as shown in Figure 2 (Rees et al., 1982). The helix of chondroitin 4-sulfate may be stabilized by chelating calcium ions between the carboxyl and 4-sulfate groups of adjacent residues (Cael et al., 1978; Nieduszynski, 1985).

As in the case of heparin and heparan sulfate, there is some controversy over the ring conformation of IdoUA in solid dermatan sulfate. From X-ray diffraction, the high helical pitch indicates that $^4C_1$ dominates (Mitra et al., 1983; Winter et al., 1986). However, molecular modeling of the homopolymer (-I-$H_{NAc,4S}$-)$_m$ with iduronic acid

TABLE 5
Helical Structures of GAGs (Arnott et al., 1974, Atkins and Nieduszynski, 1976; Cael et al., 1978; Rees et al., 1982; Millane et al., 1983; Mitra et al., 1983; Scott et al., 1983; Nieduszynski, 1985; Kvam et al., 1992)

GAG	Helix symmetry	Rise per disaccharide in nm (counterion)
Heparin (Na^+)	2_1	0.82–0.84 (Na^+ or Ca^{2+}), 0.87
HS	2_1	0.93 (Na^+), 0.84 (Ca^{2+})
Dermatan sulfate	2_1	0.94–0.97 (Na^+)
	3_2	0.95 (Na^+)
	8_3	0.92–0.93 (Na^+)
Chondroitin 4-sulfate	2_1	0.98 (Ca^{2+})
	3_2	0.94–0.96 (Na^+)
Chondroitin 6-sulfate	2_1	0.93 (low pH)
	3_2	0.95–0.96 (Ca^{2+})
	8_3	0.98 (Na^+)
Keratan sulfate	2_1	0.95 (Na^+)
Hyaluronic acid	2_1	0.98 (or low pH Na^+)
	3_2	0.94–0.95 (Ca^{2+})
	4_3	0.84, 0.93–0.97 (Na^+)
	4_3 Double helix	0.82 (and low pH K^+)

attaining $^4C_1$, $^1C_4$, and ^2T_O forms (Figure 3) showed that there is no energetic advantage of the $^4C_1$ state and that all three conformers must be taken into account (Ferro et al., 1989). Also, solid state ^{13}C NMR showed a conformation similar to $^1C_4$, contradictory to the diffraction data (Winter et al., 1986).

To resolve this problem, Venkataraman et al. (1994) have included other uronic acid conformers to screen for dermatan sulfate geometries which are compatible with both diffraction and NMR data. Their results indicate that the $^OT_2$ twist boat conformer fulfills these criteria, and that its nonbonded energy is close to the $^4C_1$ and ^2T_O con-

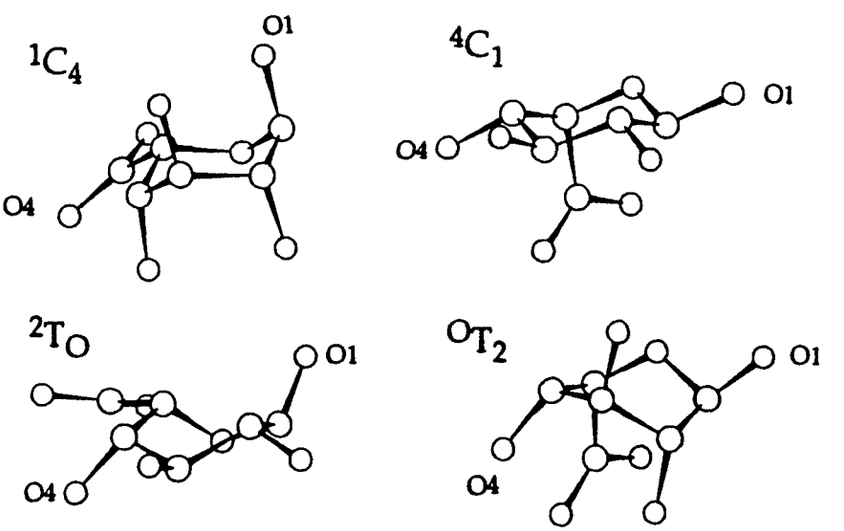


FIGURE 3. The four main conformers of α-L-iduronic acid, $^1C_4$, $^4C_1$, ^2T_O, and $^OT_2$. For simplicity, only carbon and oxygen atoms are shown.

formers. Furthermore, there is an extraordinary geometric similarity between GlcUA ($^4C_1$) and IdoUA ($^2T_0$), both at the monomer level and at the polymer level (the similarity was evaluated from the orientation of charged side groups relative to the sugar plane and the twofold helix, respectively). This indicates that if IdoUA ($^2T_0$) was an important conformer in dermatan sulfate, there would be very little difference in the morphology of dermatan sulfate and chondroitin 4-sulfate. Since these molecules have very distinct properties, for instance with respect to HCII binding and self-aggregation, $^2T_0$ is not a likely conformer. Venkataraman et al. (1994) propose that it is essential to include several monomer conformers in the search for molecular models of GAG polymers, and do the energy minimization at the polymer level instead of at the disaccharide level.

In *solution*, both chondroitin 4-sulfate and chondroitin 6-sulfate were resistant to periodate oxidation, indicating that some structure must persist (Scott, 1992). NMR studies and computer simulations have shown that they are both in the 2_1 helix form (Scott et al., 1983; Ferro et al., 1989; Scott, 1992; Scott et al., 1992), and that a dimethyl sulfoxide solution of chondroitin 4-sulfate was stabilized by three hydrogen bonds per disaccharide (Scott et al., 1983) (Figure 2).

3. Intermolecular Associations

The defined secondary structure of chondroitin sulfate in solution has made it possible to qualitatively explain its self-association properties. Chondroitin 6-sulfate, but not chondroitin 4-sulfate, can form aggregates of two or more chains which was demonstrated by rotary SEM, showing network formation in a salt-free aqueous solution (Scott et al., 1992). Molecular models showed that the twofold helix of chondroitin 6-sulfate locates the charges near the periphery and creates stretches of hydrophobicity, which can interact with similar hydrophobic patches on other chains. For chondroitin 4-sulfate, the sulfates are centered closer to the helical axis, diminishing this amphipathicity and ability to self-aggregate (Scott et al., 1992). These observations suggest that aggregation of GAGs may be controlled by the location of sulfate groups.

Dermatan sulfate fractions with alternating IdoUA and GlcUA residues can self-associate under physiological conditions and in the absence of divalent cations (Fransson and Cöster, 1979). The affinity for immobilized dermatan sulfate with 75% IdoUA is highest for dermatan sulfate with 50% iduronic acid content, but also chondroitin 4-sulfate binds to the immobilized dermatan sulfate (Fransson, 1985; Nieduszynski, 1985). Thus, chondroitin 4-sulfate cannot self-aggregate, but it does associate to dermatan sulfate.

The differences in modifications among chondroitin and dermatan sulfate determine which solid state structures are attainable and whether self-aggregation is possible. Both chelation of cations and hydrogen bonding seem to be intricately involved in determining solid state structure and extent of self-aggregation. This suggests that modification patterns contain biological information, especially for dermatan sulfate; the residues are sequestered in a non-random way along the chain, and clusters of the rare residue I_{2S} determine the HC II binding properties of the chain.

D. Keratan Sulfate

The basic repeating residue of keratan sulfate is

$$Glc_{NAc} — (\beta 1,3) — Gal\ (\beta 1,4)$$

It is the only GAG which does not contain uronic acid, preventing eliminative cleavage of keratan sulfate (Section III.A). Almost all glucosamine and some galactose units are 6-sulfated, except for keratan sulfate from erythrocyte surfaces, which has only a few sulfates (Stuhlsatz et al., 1989).

Two types of keratan sulfate are defined by their different linkage regions (Figure 4). Both are branched, which is different from linkage regions of other GAGs (Fransson, 1985; Poole, 1986; Kjellèn and Lindahl, 1991).

The molecular weight is typically around 2 to 20 kDa (Fransson, 1985). Porcine type I keratan sulfate ranges from 10 to 26 kDa (Oeben et al., 1987), and type II keratan sulfate is around 5 kDa (Fransson, 1985). The molecular weight profile of keratan sulfate is distorted toward the short

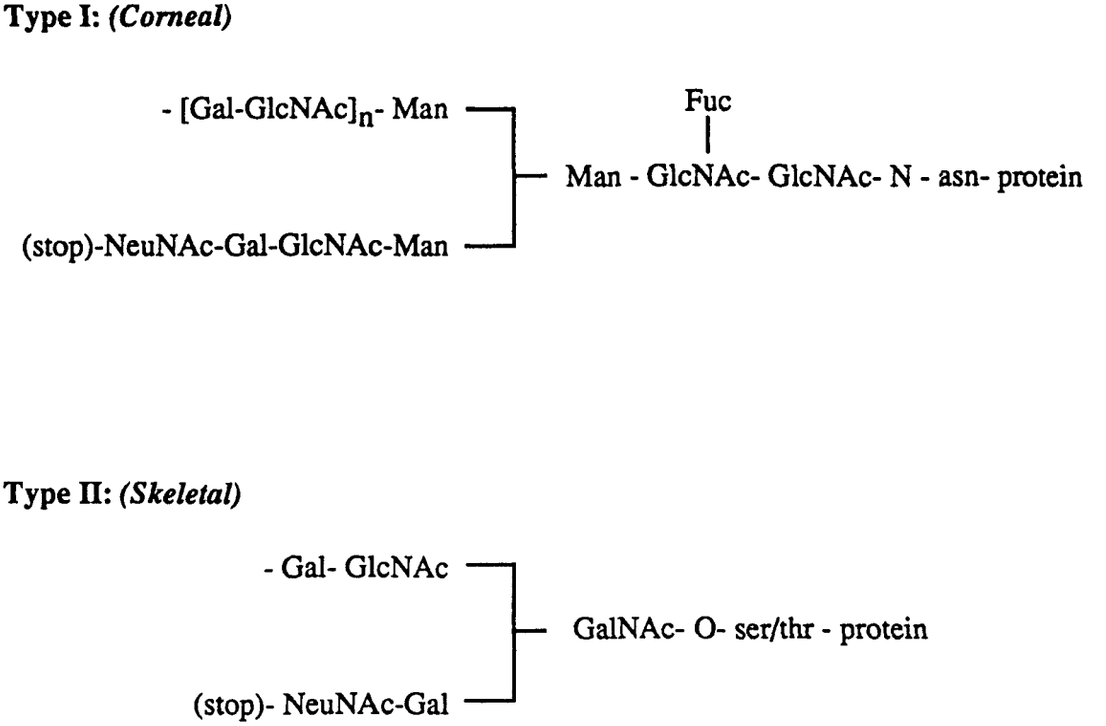


FIGURE 4. Two types of keratan sulfate are defined by their linkage regions.

chains, suggesting that some mechanism exists to control chain length either by stopping chain elongation or by enzymatic cleavage at a specific length (Stuhlsatz et al., 1981; Oeben et al., 1987). The disulfated stretch at the non-reducing end may be such a determinant for chain length (Oeben et al., 1987). An oligosaccharide, with sialic acid as nonreducing terminus, isolated from femor head cartilage, also could serve as a chain terminating signal (Nieduszynski et al., 1990).

1. Primary Structure

The overall primary structure of keratan sulfate was elucidated by enzymatic analysis. Keratanase from *Pseudomonas* sp. (Section III.B.2), which only cleaves linkages with non-sulfated galactose, was used for degradation of porcine type I keratan sulfate (Oeben et al., 1987). The products were short fragments with 0 to 1 sulfate per disaccharide, and longer fragments with close to 2 sulfates per disaccharide. The length of disulfated fragments increased with the initial length of keratan sulfate. Similar results were obtained for degradation of bovine type II keratan sulfate (Stuhlsatz et al., 1989). It was concluded that the chain must contain a few non-sulfated residues after the linkage region, followed by a monosulfated region of about 10 to 12 disaccharides, and a disulfated region of 7 to 34 disaccharides (Figure 1). These results imply that the enzymes that generate sulfation of keratan sulfate are specific for different sections of the chain.

In bovine nasal and tracheal cartilage, there is only one fucose per keratan sulfate chain (Thornton et al., 1989), most likely located in the linkage region or at the nonreducing terminus. In femoral head cartilage, however, the fucose content is proportional to the length of chain fragments. NMR studies indicate that fucose must be linked (1,3) to Glc_{NAc} of the repeat unit (Nieduszynski et al., 1990). The functional importance of fucose and the coordination of sulfate and fucose modifications, however, have not yet been investigated.

2. Secondary Structure

X-ray diffraction data for corneal keratan sulfate were in agreement with a 2_1 helical conformation with a disaccharide rise of 0.95 nm (Arnott et al., 1974; Rees et al., 1982) (Table 5). The diffraction pattern showed that the residues were perturbed slightly with respect to the positions in a crystal lattice which may reflect differences in the degree of sulfation along the polymer (Arnott et al., 1974; Nieduszynski, 1985). This twofold helix is similar to the structures of chondroitin sulfate and hyaluronic acid at low pH, when their uronic acids are protonated.

In aqueous solution, NMR studies demonstrated that disulfated keratan sulfate forms a twofold helical structure (Hounsell et al., 1986; Hounsell, 1989). Also, molecular dynamics of keratan sulfate and chondroitin sulfates in water indicated twofold symmetry and suggested that the 6-sulfate of galactose in keratan sulfate was analogous to the glucuronic acid of chondroitin sulfate; thus, the distribution and orientation of charges on these two GAGs are similar (Scott, 1992).

3. Intermolecular Associations

Keratan sulfate is self-aggregating. Preliminary results from gel chromatography and molecular dynamics indicate that monosulfated keratan sulfate is more likely to aggregate than disulfated keratan sulfate (Scott, 1992).

In summary, keratan sulfate has less variation in its primary structure than heparan sulfates and chondroitin sulfates. The chain is segregated in a monosulfated and a disulfated region, but so far no specific patterns with a higher information content have been observed. The branched linkage region and the presence of sialic acid and fucose are characteristics of keratan sulfate found in the saccharide chains of many glycoproteins, but not among the other GAGs. In polysaccharides, sialic acid and fucose often lead to highly specific protein binding (Varki, 1993); however, keratan sulfate has not, to our knowledge, been found to bind specifically to any protein (Greiling and Scott, 1989). Thus, keratan sulfate contains functional groups with the potential to form specific binding sites for proteins, and we predict that proteins that bind specifically to keratan sulfate exist, but have not yet been discovered.

E. Hyaluronic Acid

Hyaluronic acid is not sulfated and is homogeneous in its primary structure. The monosaccharides are the same as in unmodified heparin; however, the glycosidic linkages are similar to those of chondroitin sulfate. The main structural unit is:

$$Glc_{NAc} — (\beta 1,4) — GlcUA — (\beta 1,3)$$

Hyaluronic acid is considerably larger than the other GAGs, with chain lengths from 500 to several thousand disaccharides (molecular weight 100 to 1000 kDa) (Fransson, 1985).

Hyaluronic acid has not been observed covalently bound to proteins, so there is no core protein precursor to serve as a starting point for biosynthesis. As opposed to chondroitin sulfate, hyaluronic acid synthesis is not affected by drugs that inhibit protein synthesis (puromycin) or ionic transport across Golgi membranes (monensin) Evered and Whelan, 1989. These results suggest a different, and as yet unknown, biosynthetic pathway (Prehm, 1983 and 1984; Fransson, 1985).

1. Secondary Structure

Hyaluronate, which lacks the bulky sulfate-substitutions of other GAGs, shows considerable structural variability and can crystallize in several different helical forms (Table 5). Counterion, pH, and humidity determine the symmetry and disaccharide rise for a given diffraction experiment. These observations, can be rationalized at least partially, in terms of hydrogen bonding and ionic interactions. Molecular modeling indicates that in vacuo, helices of all three symmetries can form, but in water, only the twofold helices appear stable (Scott et al., 1991). This is consistent with an observed shift from fourfold to twofold symmetry when sodium salts of hyaluronate are hydrated (Guss et al., 1975). NMR studies, focusing at the protons involved in hydrogen-bonding, show that

in dimethyl sulfoxide solution the structure has four hydrogen bonds (Figure 2), but in aqueous solution the hydrogen bonding is much weaker (Heatley and Scott, 1988; Kvam et al., 1992). When calcium is present it can bind between the carboxylate groups of adjacent chains in the 3_2 form (Rees et al., 1982).

In solution, hyaluronate appears as a polymer with "stiff" sections, as demonstrated by resistance to periodate oxidation and hydrodynamic properties such as onset of non-Newtonian solution behavior at low concentration, indicating expanded chains (Morris et al., 1980; Rees et al., 1982; Heatley and Scott, 1988; Scott et al., 1991; Kvam et al., 1992). Ca^{2+} and pH have a profound effect on physical properties of hyaluronate (Gabriel and Carr, 1989). At Ca^{2+} concentrations below 3 m*M* (physiologic conditions ~5 m*M*), the solution is viscous, almost a gel, while at higher concentrations it becomes much thinner. Titration studies show that 5 m*M* Ca^{2+} or pH below 3 induces a more structured state of hyaluronic acid, possibly due to protonation of the carboxyl group (Gabriel and Carr, 1989).

2. Intermolecular Associations

Heatley and Scott discovered that the structure in aqueous solution contains a hydrophobic stretch of 8 carbon atoms on one side of the helix (Heatley and Scott, 1988), and this amphipathicity of the twofold helix may provide a hydrophobic driving force for enhancing self-association of hyaluronate (Scott, 1992). Interchain association of hyaluronic acid into a double helix has been demonstrated by X-ray diffraction at acidic pH in the presence of potassium and other counterions, but not with sodium (Sheehan et al., 1977). In this structure, the partially protonated carboxylic acids are in the center of the double helix, possibly chelated by the counterion.

Large aggregates of hyaluronic acid with proteoglycans of keratan sulfate and chondroitin sulfate are found in cartilage (Poole, 1986). The proteoglycans of these aggregates are very tightly, but noncovalently, bound to hyaluronate of at least nine saccharides length (K_d = 20-30 n*M*) (Nieduszynski, 1985). High salt concentrations (>0.15 *M* NaCl) reduce the effective charge-repulsion between the polymers and enhance hyaluronic acid aggregation (Scott et al., 1991).

Thus, hyaluronic acid is the only GAG that has no sequence variability; however, the secondary structures are very diverse. The environment appears to have a pronounced effect on hyaluronic acid structure.

F. Structural Features of GAGs

The glycosaminoglycans as a class have similar backbone residues, which distinguish them from other polysaccharides. Modification of these backbone residues can vary considerably, even among GAGs belonging to the same family. The backbone is modified after polymerization (Lindahl, 1989), which could lead to at least some randomness in the sequence compared with the template biosynthesis of DNA or proteins. However, in this review we propose that the primary sequence of modified residues in GAGs has two levels of organization.

On a macroscopic level, it has been demonstrated that heparan sulfate and dermatan sulfate are segmented into alternating blocks of specific sulfation patterns (Figure 1). Also keratan sulfate is segregated, but in a manner where the degree of sulfation increases in two steps as one moves away from the core protein. It is not yet clear whether these increases occur from one residue to the next, or if there is a gradient of sulfation in a transition region between the blocks.

On a microscopic level, at least four different GAG sequences have been associated with specific binding functions. The AT III binding pentasaccharide of heparin, the bFGF binding pentasaccharide of heparan sulfate, the 2-sulfated glucosamine of heparan sulfate which direct nuclear localization, and the HCII binding hexasaccharide of dermatan sulfate (Figure 1) all contain information in their primary sequence that directs their biological function (Lindahl et al., 1984; Isihara et al., 1986; Maimone and Tollefsen, 1990; Maccarana et al., 1993).

These two levels of organization are of utmost importance in controlling the physical form of GAGs and regulating the specific interactions between two GAGs and between a GAG and a protein. Additionally, the physical form is dependent on the ionic environment, which shields the

repulsive ionic forces between the charged chains. Secondary structure of GAGs in solid state is often highly dependent on the counterions present in the fibers or crystals (Cael et al., 1978; Rees et al., 1982; Millane et al., 1983) (Table 5). Calcium and high ionic strengths facilitate secondary structure formation in fiber diffraction experiments. Stabilization of the helices seems to be dominated by hydrogen bonds between functional groups from adjacent residues (Rees et al., 1982) suggesting that the helix-forming potential is determined by the primary structure. The primary structure also can determine whether dimeric or higher aggregates of GAGs can form. Only heparan sulfate and dermatan sulfate with the right degree of glucuronic acid epimerization can self-associate; and helices of chondroitin 6-sulfate show amphipathicity, allowing self-association, as opposed to chondroitin 4-sulfate, which has little or no amphipathicity (Nieduszynski, 1985; Gallagher et al., 1986; Scott, 1992).

In solution, heparin, heparan sulfate, and dermatan sulfate appear to be very flexible molecules, while chondroitin sulfates and hyaluronate, which do not contain IdoUA, appear to have a certain "stiffening of the chain' and form hydrogen bonds between adjacent residues in aqueous solutions (Morris et al., 1980; Rees et al., 1982; Scott et al., 1983; Scott, 1992; Scott et al., 1992). The sugar ring of the IdoUA residue can attain at least three different puckers, which under physiological conditions are energetically very close (Ragazzi et al., 1986; Ragazzi et al., 1990; Venkataraman et al., 1994). Interconversion among these states may generate conformational flexibility in the chains which facilitates interaction with proteins (Rees et al., 1985; Casu et al., 1986). We propose that specific interactions with binding proteins and enzymes are governed by the local formation of secondary structure of the GAG chain, which in turn is determined by the disaccharide sequence modifications.

G. GAG-Protein Interactions

A wide variety of ECM-, intracellular, and plasma proteins bind to GAGs (Cardin and Weintraub, 1989; Jackson et al., 1991). Binding often has been found to be specific for heparin or heparin oligosaccharides, even in comparison to binding to oversulfated GAGs of the same charge density as heparin (Ogamo et al., 1985; Lee and Lander, 1991; Lortat-Jacob and Grimaud, 1992; Coltrini et al., 1993). Additionally, protein sequence domains enriched in basic amino acids are often required for binding (Cardin et al., 1991; Jackson et al., 1991). Protein crystal structures have demonstrated that these protein sequence domains, in the case of AT III and bFGF, are brought close enough by folding to form a single binding domain on the protein surface (Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991; Mourey et al., 1993; Zhu et al., 1993; Schreuder et al., 1994). The protein structure enables molecular modeling of the binding interactions, which has qualitatively highlighted amino acids of the protein and functional groups of the oligosaccharide that are in close contact and are the main contributors to the binding energy (Grootenhuis and van Boeckel, 1991; Grootenhuis et al., 1994; Thompson et al., 1994).

In view of the close contacts between functional groups proposed for protein-GAG complexes, the local secondary structure of the GAG chain must be geometrically complementary to the binding pocket of the protein. One can imagine the solution state of the GAG chain as being a dynamic equilibrium among many energetically close geometries. When binding to a protein, like FGF or AT III, the chain attains the geometry that allows the most favorable binding interactions with the more conformationally rigid protein. Ornitz and Waksman co-crystallized bFGF with a heparin-derived trisaccharide (**I-H_{NAc}-G**) and found that the growth factor dimerized with four trisaccharides bound per dimer (Ornitz et al., 1995). The glucosamine sugar pucker was different in the different binding sites (Waksman, personal communication); this is strong support for the hypothesis that heparin is flexible in solution, and adapts to a conformation that complements the binding pocket of a protein on binding. We propose that this paradigm is the key to understanding the specificity in GAG-protein interactions.

H. GAGs in the Extracellular Matrix

GAGs are ubiquitous, soluble components of the extracellular matrix. The physical form of

GAGs and the specific interactions between GAGs and other ECM components are often directed by the sequence of disaccharide residues.

1. Physical Form of GAGs in ECM

In the extracellular matrix, the aggregation of GAGs often involve interaction between GAGs and proteins and between two GAGs from different classes (Hardingham and Fosang, 1992). In cartilage, the proteoglycans of chondroitin sulfate and keratan sulfate aggregate with hyaluronic acids through their core proteins. X-ray fiber diffraction studies of this aggregate indicate that GAGs from different proteoglycans interact, possibly via divalent cations (Rees et al., 1982). Another example of these protein-GAG interactions for aggregate formation is from the corneal stroma, where the core proteins of dermatan sulfate PG bind to collagen fibers. The space between fibers corresponds to the length of a typical dermatan sulfate chain, and this distance is conserved in more than 30 animal species (Scott, 1992). It was speculated that dermatan sulfate self-association controls the distance between collagen fibers, thus enabling the crucial transparency of the cornea (Scott, 1992).

Most often, the GAG depolymerizing enzymes are investigated with solutions of purified substrate; this is a very different environment from the complex extracellular matrix (Evered and Whelan, 1986; Hay, 1991; Hardingham and Fosang, 1992; Zern and Reid, 1993). Thus, it must be kept in mind that the GAG depolymerizing enzymes probably have evolved to cleave GAGs in their natural state, and that studies of soluble substrates may provide only part of the key to understand the enzymatic mechanism.

2. Specific Interactions of GAGs with Transient Components of ECM

In addition to binding of AT III, FGF, HC II, and other molecules already mentioned, GAGs also appear to be involved in such diverse processes as sequestering of superoxide dismutase (Sandström et al., 1992), covalent crosslinking of the blood protein pre-α-inhibitor (Enghild et al., 1991), L-selectin binding (Nelson et al., 1993; Norgard-Sumnicht et al., 1993; Norgard-Sumnicht and Varki, 1995), and as microbial and viral attachment sites (Shieh et al., 1992; Ascencio et al., 1993; Isaacs, 1994). This impressive list of specialized functions emphasizes the importance of structural diversity of GAGs.

In summary, GAGs are found in the extracellular matrix either bound to structural proteins like collagen, laminin and fibronectin, aggregated with other proteoglycans, or as part of integral membrane proteoglycans (Hardingham and Fosang, 1992) (Figure 5). The functions of GAGs range from providing structure and hydration to the matrix to modulating ECM processes specifically. Although certain GAGs have been observed to localize to the nucleus of hepatocytes (Fedarko and Conrad, 1986), GAGs typically interact with transient components of the matrix, either at the cell surface, in solution or sequestered in the matrix. For example, bFGF-induced endothelial cell proliferation requires trimolecular interactions between bFGF, FGF-receptor and heparan-sulfate (Kiefer et al., 1991; Klagsbrun and Baird, 1991; Yayon et al., 1991; Jaye et al., 1992). This interaction activates tyrosine kinase activity of the intracellular domain of the FGF receptor, which in turn leads to secondary messenger activation. Recent studies have added an extra dimension to this cell-surface event by indicating that heparan sulfate mediates dimerization, either of bFGF, of FGF-receptor or of both which may be a necessary part of signal transduction (Ornitz et al., 1992; Mascarelli et al., 1993; Pantoliano et al., 1994; Springer et al., 1994; Ornitz et al., 1995).

In this section we have reviewed the literature on GAG structure and shown that the primary structure of the glycosaminoglycan is important, both for the physical form and for the specific interaction of GAGs with extracellular matrix components. We have proposed that specific interactions of GAGs with proteins can be conceptualized as the 'freezing out' of a given geometry of the oligosaccharide on binding from a flexible solution state that involves dynamic equilibrium among several geometries.

III. ENZYMES

In vivo, GAGs are degraded by hydrolases (EC 3.2.1.-), such as heparin hydrolase, keratanase, hyaluronic acid hydrolase and various exogly-

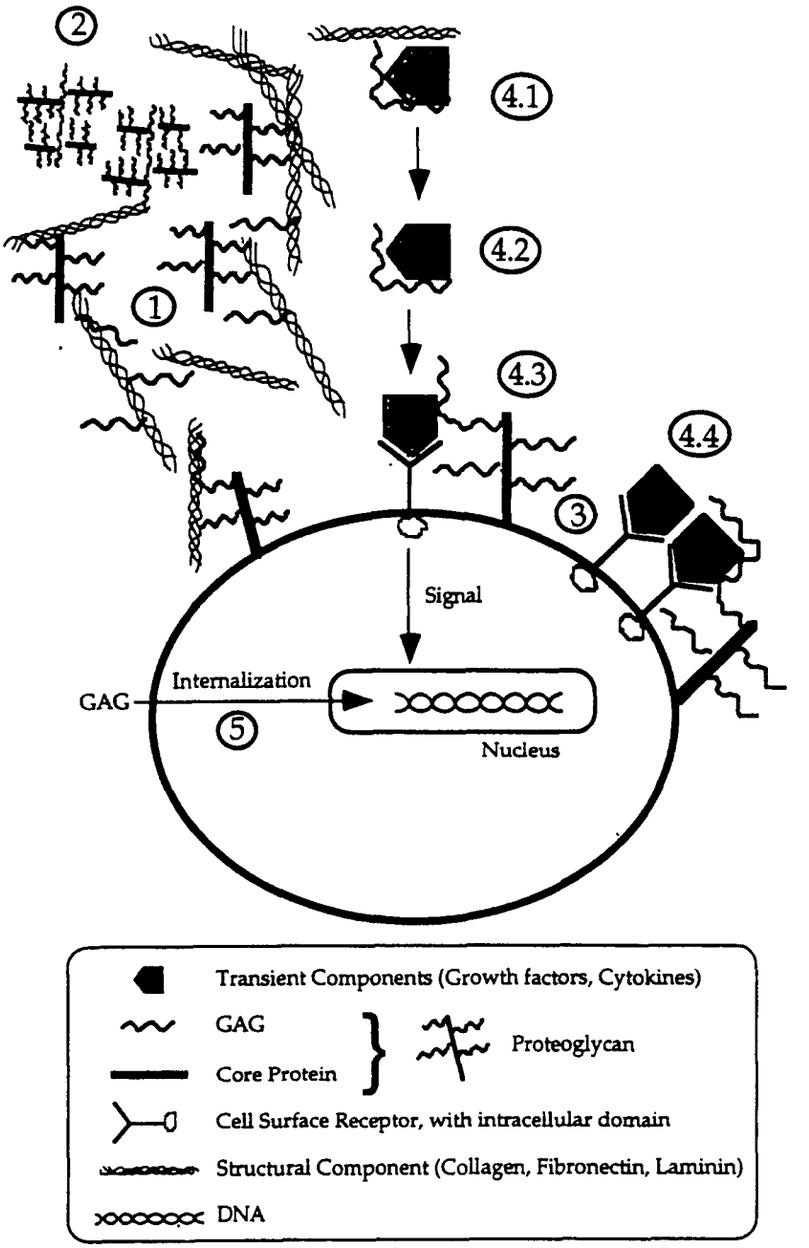


FIGURE 5. Localization and function of GAGs in the extracellular matrix. GAGs are found bound to structural components of ECM (1), aggregated in large proteoglycan complexes (2), or at cell surfaces as integral membrane proteoglycans (3). Their functions include binding to transient components of the matrix either sequestered in the matrix (4.1), as soluble complexes (4.2), or at cell surfaces as co-receptors for cell-stimulating signals (4.3), possibly involving dimerization of growth factor and/or receptor (4.4), and also internalization and localization to the nucleus (5)

cosidases (Hopwood, 1989; Kresse, 1989; Roden et al., 1989). There also are three categories of GAG degrading lyases mainly of bacterial origin: heparinases, chondroitinases and hyaluronidases (Linhardt et al., 1986). These lyases have been purified and utilized for analytical and industrial

applications; as a consequence, they are more thoroughly characterized than the corresponding hydrolases. They belong to a class of polysaccharide lyases (EC 4.2.2.-) that also includes alginases, several pectinases, and xanthan lyase. These enzymes resemble the GAG lyases in some respects, and for this reason they are included in this review.

This section focuses on the properties and mechanisms of the enzymes that depolymerize glycosaminoglycans. The sources, fermentation and purification of GAG lyases (Linhardt et al., 1986) and hydrolases (Evered and Whelan, 1989; Greiling and Scott, 1989; Lane and Lindahl, 1989) are reviewed elsewhere.

The chemistry of eliminative cleavage by lyases and hydrolytic cleavage by hydrolases is illustrated in Figure 6. The difference is that in the eliminative mechanism the C5 hydrogen of uronic acid is abstracted, forming an unsaturated C4-C5 bond, whereas in the hydrolytic mechanism a proton is donated to the glycosidic bond, breaking the glycosidic oxygen and creating an O5 oxonium ion followed by water addition, which neutralizes the oxonium ion and saturates all carbons (Linhardt et al., 1986). The lyases can only cleave linkages on the non-reducing side of uronic acids, as the carboxylic group of uronic acid participates in the reaction. The hydrolases, on the other hand, can be specific for either of the two bonds in the repeating disaccharides.

Specialized techniques have been employed to study the properties of GAG degrading enzymes. A simple measurement of product formation is based on the 232 nm absorbance of the Δ4,5 unsaturated bond of GAG cleavage by lyases (Linker and Hovingh, 1972). The details of kinetic mechanism is studied by evaluation of the product profile from various substrates by gel-electrophoresis, HPLC and capillary zone electrophoresis (CZE), and by identification of the various products by two-dimensional NMR; this has allowed very accurate characterization of substrate specificity and reaction kinetics (Rice and Linhardt, 1989; Linhardt et al., 1990).

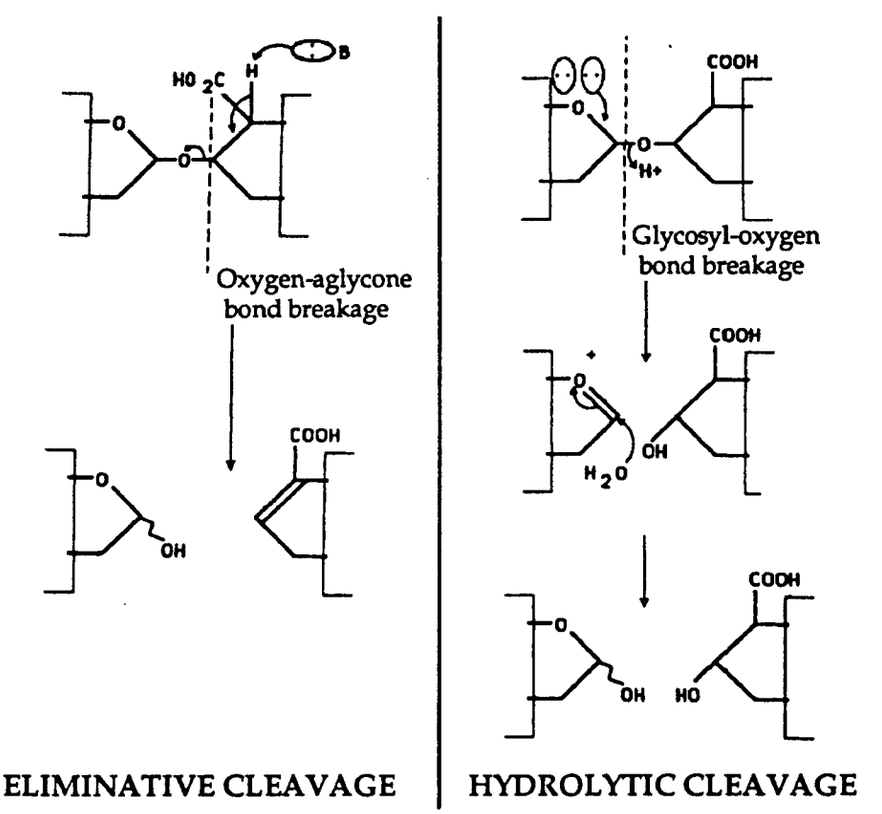


FIGURE 6. Eliminative and hydrolytic cleavage of GAGs. Modified with permission (Linhardt et al., 1986).

A. Lyases

The GAG degrading lyases are divided in three categories according to their primary substrate: heparinases, chondroitinases and hyaluronidases. Within each category there is a further subdivision of the enzymes based on details of their specificities.

1. Heparinases

Six enzymes that cleave heparin or heparan sulfate have been isolated from four different bacteria: *Flavobacterium heparinum, Bacillus* sp., *Bacteroides heparinolyticus*, and an unclassified soil bacterium. At least twenty-nine other bacteria express heparinase activity as determined by a qualitative plate assay (Salyers et al., 1977; Steyn et al., 1992); however, these enzymes have not been isolated or characterized. The enzymes have been classified as heparinase I acting primarily on heparin, heparinase II acting on both heparin and heparan sulfate, and heparinase III, which acts exclusively on heparan sulfate (McLean et al., 1985). The main properties of the six known heparinases are summarized in Table 6.

Most heparinase-producing bacteria were found in soil. The biological advantage for soil bacteria of expressing heparinase and other acidic polysaccharide lyases is unknown, although it is likely that bacteria with these enzymes can degrade glycosaminoglycans from carcasses. Heparin, heparan sulfate, or fragments thereof induce heparinase production in several strains, and expression in *F. heparinum* can also be derepressed by sulfur deprivation (Cerbelaud et al., 1986).

The heparinases have been purified to single (although in the case of enzymes from *F. heparinum* broad) bands on Coomassie stained SDS-PAGE (Yang et al., 1985; Nakamura et al., 1988; Böhmer et al., 1990; Bellamy and Horikoshi, 1992; Lohse and Linhardt, 1992). Monoclonal antibodies toward heparinase I had equally high affinity for heparinase I and II, but 10 to 100 fold lower affinity for heparinase III (Gu et al., 1993a). Antibody assays and Southern blotting showed no cross reactivity between heparinases from different bacteria (Bellamy and Horikoshi, 1992; Sasisekharan et al., 1993). These results indicate that there is a low degree of sequence and structural homology among the heparinases.

Isoelectric focusing showed that *Bacillus* sp. heparinase (pI = 6.6) has an overall *negative* charge at its optimal pH of 7.5, while all three *F. heparinum* heparinases (pI = 8.5-10.1) are positively charged at their pH optima of 7-7.5. For enzymes that degrade a polyanionic substrate, this appears to be an important difference that may imply different mechanisms for heparinases from *Bacillus* sp. and from *F. heparinum*.

Only the gene for heparinase I from *F. heparinum* has been cloned and sequenced (Sasisekharan et al., 1993), and the recombinant gene product purified to homogeneity (Ernst et al., 1995). For the other heparinases, the amino acid composition was determined by acid hydrolysis (Yang et al., 1985; Bellamy, 1990; Böhmer et al., 1990; Lohse and Linhardt, 1992). Although the *Bacillus* sp. heparinase has a low pI, it contains around 12% basic amino acids, which is similar to heparinase II, III, and heparnase from the unclassified strain, which all have much higher pI. The key to this difference probably lies in the contents of acidic amino acids. Unfortunately, asparagine and glutamine are deamidized by acid treatment during amino acid analysis, thus, disguising the original content of aspartic acid and glutamic acid.

a. Reaction Conditions

For heparinase I, II, and III from *F. heparinum* the optimal temperature for exhaustive degradation assays (~10 to 100 h) is 5 to 10°C less than for short degradation assays (10 to 30 min) (Lohse and Linhardt, 1992). Similarly, the temperature optimum for *Bacillus* sp. heparinase is 50°C, but 90% of the activity is lost after 3 h at 45°C (Bellamy and Horikoshi, 1992). For short assays, sodium phosphate buffer has been reported to give higher activity than sulfonate-based buffers (MOPS, TES, and HEPES), but the latter seem to increase stability of heparinase I, II, and III (Lohse and Linhardt, 1992). It appears, in general that the optimal conditions for heparinases are different

TABLE 6
Properties of Heparinases from Various Sources

	Heparinase I				Heparinase II	Heparinase III
Source	*Flavobacterium heparinum*	*Bacillus sp*	*Bacteroides heparinolyticus*	*Unclassified*	*Flavobacterium heparinum*	*Flavobacterium heparinum*
Characteristics	Soil Gram-negative periplasmic space inducible	Soil Gram-positive secreted inducible	Mouth Gram-negative cell-associated inducible	Soil Gram negative cell-associated constitutive	Soil Gram-negative periplasmic space	Soil Gram-negative periplasmic space
Molecular properties						
Molecular weight	41,700[a]	116,000	63,000	94,000	84,100	70,800
pI	8.5–9.3	6.6	9.5	9.3	8.9–9.1	9.9–10.1
Amino acid composition						
mole% lysine	11.2	6.0		8.0	6.5	6.3
mole% arginine	3.1	5.8		2.7	5.1	5.5
mole% cysteine	0.5	0.4		0.1	0.4	0.0
mole% hydrophobic	36.5	46.5		42.9	38	36.8
Activity						
optimum for assays of 10–30 min	35°C	50°C	<<45°C	45°C	40°C	45°C
pH optimum	6.5–7.15	7.5	6.5	7,6	6.9–7.3	7.6
Optimum (*M*)	0.1–0.2			0.15	<0.05	<0.05
Specific activity (IU/mg)	197–219 (heparin)	5–24 (heparin)		127 (heparin)	19 (heparin) 36.5 (HS)	63.5–141 (HS)
Turnover number (s^{-1})[b]	140	10–46		200	27–51	75–166
K_m (μM disaccharide)	7 to 440[b]		65[b]	1400 (heparin) 480 (HS)	1300 (HS)	
Relative initial rate						
Heparin	100%	100%	100%	100%	100%	~0
HS	20–28%	30%	65%[b]	11%	192%	100%
Additives						
Inhibitors	EDTA, Hg^{2+}, Cu^{2+} polyvinylsulfate, sulfonate buffer IAA (1 m*M*)	Cu^{2+}, Cu^{+}, Zn^{2+}, Fe^{3+}, Hg^{2+}, Co^{2+}, Ni^{2+}	Cu^{2+}, Hg^{2+}		Ca^{2+} (10 m*M*), Hg^{2+}(10 m*M*), Li^{+}, Zn^{2+}, Ba^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} (1 m*M*)	Zn^{2+}, Hg^{2+}, Cu^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+} (10 m*M*)
Compounds with no effect	β-ME	EDTA (1 m*M*), β-ME (1 m*M*), IAA (1 m*M*)	Ca^{2+} (0.2 m*M*), EDTA (0.1 m*M*), β-ME (1 m*M*)			
Activators	Ca^{2+} (1 m*M*)	Ca^{2+} (5 m*M*), Mg^{2+}, Ba^{2+}	Fe^{2+} (1 m*M*)			slightly activated by Ca^{2+} (10 m*M*)
Commercially available	+				+	+

Ref.	(Yang et al., 1985; Rice and Linhardt, 1989; Linhardt et al., 1990; Nader et al., 1990; Ampofo et al., 1991; Sasisekharan, 1991; Lohse and Linhardt, 1992; Sasisekharan et al., 1993)	(Bellamy and Horikoshi, 1992; Bellamy, 1990)	(Nakamura et al., 1988)	(Joubert and Pitout, 1985; Böhmer et al., 1990)	(Silva et al., 1976; Linhardt et al., 1990; Nader et al., 1990; Lohse and Linhardt, 1992)	(Silva et al., 1976; Linhardt et al., 1990; Nader et al., 1990; Lohse and Linhardt, 1992)

[a] Deduced from DNA sequence.

[b] Apparent values for K_m were reported based on Lineweaver-Burke plots of the initial rate of product formation versus the initial substrate concentration and have been converted into molar concentration on a disaccharide basis, assuming a mol wt of 573 and 458 for disaccharides from heparin and HS, respectively. The turnover number is calculated for enzymes that have been purified to homogeneity.

for experiments running over several hours or days compared with short assays. Lower temperature, sulfonate buffers, and possibly higher ionic strength may increase stability during long assays, but this question remains controversial.

b. Kinetic Constants

The specific activities of the different purified enzymes are similar at 50 to 200 IU/mg. Only heparinase II from *F. heparinum* and heparinase I from *Bacillus* sp. seem to have lower specific activities. For heparinase I from *F. heparinum*, Yang et al. report a V_{max} of 197 IU/mg for substrate concentrations up to 5.5 mg/ml, but 68 IU/mg for 24 mg substrate/ml (Yang et al., 1985), suggesting that substrate inhibition may be significant (Bailey and Ollis, 1986). The large variation in K_m for heparinase I may reflect the difficulties in fitting standard Michaelis-Menten kinetics to degradation of complex polymeric substrates. The oligosaccharide products of initial degradation will themselves be substrates for further degradation, thus increasing the molar concentration of substrate. Also, the K_m determinations were based on different ranges of heparin concentrations; this may have biased the reports.

Calcium at 1 to 5 m*M* is a necessary activator for heparinase I (Linker and Hovingh, 1972; Sasisekharan, 1991), while heparinase II is inhibited by 10 m*M* calcium (Bellamy, 1990; Lohse and Linhardt, 1992). In some studies, little or no activation of heparinase I by calcium was observed (Dietrich et al., 1973; Linhardt et al., 1986; Lohse and Linhardt, 1992), but in these experiments, trace contaminations from hydroxylapatite columns or chelated substrates may have provided the necessary Ca^{2+} for activity. In separate circular dichroism (CD), NMR and equilibrium dialysis experiments, it was found that calcium binds to heparin, thereby changing its structure (Rees et al., 1982; Hunter et al., 1988). In light of that observation, the different effect of calcium on activity of heparinases is very interesting. It is possible that heparinase I is specific for a different, calcium dependent, structure than heparinase II.

c. Substrate Specificities of Heparin Lyases

The substrate specificity of heparin lyases has been defined as a disaccharide containing a cleavable linkage. Heparinase I cleaves the linkages $H_{NS,6X}$-I_{2S}, heparinase II the linkages $H_{NY,6X}$-G/I_{2X}, and heparinase III the linkages H_{NAc}-I and $H_{NY,6X}$-G, in which Y is sulfated or acetylated and X is sulfated or unsubstituted. The experimental background for these conclusions is reviewed in the following.

i. Heparinase I from *F. heparinum* (E.C. 4.2.2.7)

This is mainly active toward heparin, although heparan sulfate also is degraded. The products of complete degradation of heparin have been quantitated by Linhardt and coworkers after separation by strong anion exchange (SAX) HPLC (Table 7) (Rice and Linhardt, 1989; Linhardt et al., 1990; Nader et al., 1990; Linhardt et al., 1992; Linhardt et al., 1992a). The identification of oligosaccharide products is initially done by various one- and two-dimensional NMR techniques. There is a discrepancy between the results of Linhardt and coworkers (Rice and Linhardt, 1989; Loganathan et al., 1990; Ampofo et al., 1991; Linhardt et al., 1992; Linhardt et al., 1992a) who find that the main hexasaccharide (**8**) contains one *N*-acetylated and one 3-sulfated glucosamine, and those of Gettins and Horne (1992) who find two hexasaccharides: U_{2S}-$H_{NS,6S}$-I_{2S}-$H_{NS,6S}$-G — $H_{NS,6S}$ and U_{2S}-$H_{NS,6S}$-I_{2S}-$H_{NS,6S}$-I_{2S}-$H_{NS,6S}$. The molecular weight distribution of the products in the study by Gettins and Horne suggests that their reaction was incomplete, and that the hexasaccharide fraction therefore is dominated by intermediate products with composition similar to undegraded heparin.

The "typical" tetrasaccharides (**4** and **6**), with composition corresponding to the most frequent linkages in heparin, are present among the degradation products, demonstrating that heparinase activity is dependent on the size of the substrate. When these tetrasaccharides are used as substrates, they are cleaved several orders of magnitude more slowly than polymeric heparin (Rice and Linhardt, 1989; Linhardt et al., 1990). It has not been inves-

TABLE 7
Products from Heparinase I Degradation of Porcine Heparin

Peak number	Structure	Mole %
1	ΔU_{2S}-H_{NS}	2.5
2	ΔU-$H_{NS,6S}$	0.5
3	**ΔU_{2S}-$H_{NS,6S}$**	**71.0**
3a	ΔU_{2S}-H_{NS}-I_{2S}-H_{NS}	0.8
3b	ΔU_{2S}-H_{NS}-G-$H_{NS,6S}$	0.6
4	**ΔU_{2S}-H_{NS}-I_{2S}-$H_{NS,6S}$**	**3.1**
4a	ΔU_{2S}-$H_{NS,6S}$-I-$H_{NS,6S}$	0.5
5	**ΔU_{2S}-$H_{NS,6S}$-G-$H_{NS,6S}$**	**6.9**
6	**ΔU_{2S}-$H_{NS,6S}$-I_{2S}-$H_{NS,6S}$**	**3.2**
6a	ΔU_{2S}-$H_{NS,6S}$-I-$H_{NAc,6S}$-G-$H_{NS,6S}$	0.8
7	ΔU_{2S}- $H_{NS,6S}$-G-$H_{NS,6S,3S}$	0.6
8	**ΔU_{2S}-$H_{NS,6S}$-I-$H_{NAc,6S}$-G-$H_{NS,6S,3S}$**	**2.2**

Note: The products are numbered in order of elution on SAX-HPLC (Linhardt at al., 1992b). The main di- , tetra- , and hexasaccharides are bolded. The mole % is calculated as the absorbance of the fraction divided by the total absorbance. 90.2% (w/w) of heparin substrate was recovered as products

tigated whether the hexa- and octasaccharides are equally susceptible as heparin, or if they are cleaved at some intermediate rate. Tetrasaccharides with glucuronic acid could not be cleaved, even at high enzyme concentrations, demonstrating that iduronic acid is an absolute requirement for catalytic activity. Hexosamine without 6-sulfation appears at the reducing end of degradation products **1** and **3a** (Table 7) and must therefore be cleavable, while linkages with *N*-acetylation are much less susceptible (Yang et al., 1985; Desai et al., 1993a). It is possible, that the *N*-acetylated residues of the hexasaccharides inhibit cleavage both at the reducing side and at the second bond on the non-reducing side. This could explain why *N*-acetylated residues are not found in tetrasaccharides. In summary, heparinase I is specific for linkages of the type $H_{NS,6X}$-I_{2S}, in hexa- and larger oligosaccharides (Table 8).

ii. Heparinase II from *F. heparinum*

Heparinase II, also known as heparitinase II, is active on both heparin and heparan sulfate, with the heparan sulfate activity being approximately twice as high as the heparin activity (Linhardt et al., 1990; Nader et al., 1990; Lohse and Linhardt, 1992). The distribution of products for heparinase II degradation of heparin is not as well characterized as for heparinase I. It reflects the composition of the substrate: heparin yields predominantly a trisulfated disaccharide (**3**), while heparan sulfate is degraded to mono- and disulfated disaccharides (Table 9). In addition, there are a large variety of different disaccharides present in detectable amounts. Heparin that has been chemically modified by either 2-*O* or 6-*O* desulfation, or *N*-acetylation is still a good substrate for heparinase II (Moffat et al., 1991a; Desai et al., 1993a). These findings reflect a broad substrate specificity of heparinase II.

The main tetra- and hexasaccharide products of heparinase I degradation were subjected to heparinase II treatment (Linhardt et al., 1990), and it was found that linkages with glucuronic acid and linkages with iduronic acid are equally good substrates. It is remarkable that this eliminase is capable of cleaving both of the isomeric uronic acids even though the epimerized C5 hydrogen is being abstracted in the reaction. This is unusual for enzymatic reactions which are normally stereospecific due to the defined structure of the enzyme catalytic site. It is an interesting question if heparinase II has one active site specific for iduronic acid residues and one for glucuronic acids, or if they are somehow accommodated into one

TABLE 8
Specificities of GAG Degrading Enzymes

	Cleavable linkage[a]	Minimum Length	Mode of action
Lyases			
Heparinase I	....$H_{NS,6X}$ - (α1,4) - I_{2S}....	n = 6	Mixed
Heparinase II	....$H_{NY,6X}$ - (α1,4) - U_{2X}....		Endolytic
Heparinase III	....$H_{NY,6X}$ - (α1,4) - **G**....		Endolytic
	and ....H_{NAc} -(α1,4) - **I**....		
Chondroitinase ABC	$H_{NAc,4X,6X}$ - (β1,4) - U_{2X}[b]		Exolytic
Chondroitinase AC	....$H_{NAc,4X,6X}$ - (β1,4) - **G**....[c]		Endolytic (*F. hep*), mixed (*A. aur*)
Chondroitinase B	....$H_{NAc,4X,6X}$ - (β1,4) - I_{2X}....	n = 6	
Chondroitinase C	....$H_{NAc,6S}$ - (β1,4) - **G**....		
Hyaluronidase	....H_{NAc} - (β1,4) - **G**....		
Hydrolases			
Platelet heparanase	....I_{2X} - $H_{NY,6X}$ -**G** - (β1,4) - $H_{NY,6X,3X}$... ↑	n = 8	Endolytic
Tumor heparanase	....I_{2S} - $H_{NAc,6X}$ -**G** - (β1,4) - $H_{NAc,6X}$.... ↑	n = 12	Endolytic
Keratanase I	...$H_{NAc,6X}$-**Gal** - (β1,4) - $H_{NAc,6X}$....[d] ↑		
Keratanase II	...$H_{NAc,6S}$ - (β1,3) - Gal_{6X}-$H_{NAc,6S}$... ↑		
Hyaluronidase I	....H_{NAc} - (β1,4) - **G**....		
Hyaluronidase II	....**G** - (β1,3) - H_{NAc}....		
β-glucuronidase	**G** - (β1,4)/(β1,3) - $H_{NY,6S\ or\ 4S}$....	n = 2	Exolytic
α-L-iduronidase	**I** - (a1,4)/(a1,3) - $H_{NY,4X,6X}$....	n = 2	Exolytic
β-galactosidase	**Gal** - (β1,4)/(β1,3) - $H_{NAc,6X}$....		Exolytic
α-*N*-acetylglucosaminidase	H_{NAc} (α1,4) - G_{2X}....	n = 2	Exolytic
β-*N*-acetylhexosaminidase	H_{NAc} (β1,4)/(β1,3) - G_{2X}....		Exolytic

[a] Y = acetylated or sulfated, X = sulfated or unsubstituted.
[b] Chondroitinase ABC requires at least one sulfate on the galactosamine.
[c] It is unknown if chondroitinase AC cleaves double sulfated linkages.
[d] Keratanase I requires at least one 6-sulfate on the glucosamines.

TABLE 9
Products from Degradation of Heparin and HS with Heparinase II and III

Enzyme	Heparinase II		Heparinase III
Substrate	**Heparin**	**Heparan sulfate**	**HS**
Main products	U_{2S}-$H_{NS,6S}$ $U_{2S\text{-}HNS}$ U-$H_{NS,6S}$	U-H_{NAc}, U-H_{NS}, U-$H_{NS,6S}$, U-$H_{NAc,6S}$	U-H_{NAc} U-H_{NS} oligosaccharides
Minor products	U-$H_{NAc,6S}$ U_{2S}-$H_{NS,6S}$	$U_{2S\text{-}HNAc,6S}$ $U_{2S\text{-}HNS}$	U-HNAc,6S $U_{2S\text{-}HNAc}$
Ref.	Linhardt et al., 1990; Ampofo et al., 1991; Moffat et al., 1991		Silva et al., 1976; Linhardt et al., 1990; Nader et al., 1990; Ampofo et al., 1991

site. In summary, the susceptible linkages are $H_{NY,6X}$-U_{2X}, where the uronic acid can be IdoUA or GlcUA, the X group can be sulfated or unsubstituted, and Y can be either sulfated or acetylated but not free (Table 8).

iii. Heparinase III from *F. heparinum* (E.C. 4.2.2.8)

Heparinase III, is also known as heparitinase I and is active only toward heparan sulfate. Neither SAX-HPLC chromatograms (Linhardt et al., 1990), CZE electropherograms (Ampofo et al., 1991), nor gradient PAGE (Desai et al., 1993a) show any reaction products when heparin is incubated with heparinase III. The degradation products from heparinase III degradation of heparan sulfate (Table 9) are poorly sulfated (0 to 1 sulfate/disaccharide). In a SAX-HPLC chromatogram recorded by Linhardt's group there are at least 8 minor peaks following the main products (Linhardt et al., 1990). They are probably oligosaccharides from the *N*-sulfated regions of heparan sulfate (Figure 1).

Incubation of heparinase III with oligosaccharides from enzymatic and chemical degradation of heparin and heparan sulfate showed that it is inactive towards linkages containing iduronic acid with or without 2-*O*-sulfation, but cleaved tetrasaccharides with glucuronic acid (Silverberg et al., 1985; Rice and Linhardt, 1989; Linhardt et al., 1990) It is more active toward larger oligosaccharides than toward smaller. It is active toward oligosaccharides with *N*,6-disulfated or *N*-acetylated hexosamines (Rice and Linhardt, 1989; Linhardt et al., 1990), indicating that *N*-sulfation and 6-*O*-sulfation does not inhibit cleavage. This is confirmed by the existence of 6-*O*-sulfated and *N*-sulfated products after cleavage of polymeric heparan sulfate (Table 9).

Recently, it was reported that heparin, which had been *O*- and *N*-desulfated and *N*-acetylated, could be cleaved by heparinase III producing a non-sulfated disaccharide corresponding to 67% of the mass of starting material (Desai et al., 1993a). This implies that unsulfated linkages with iduronic acid can be cleaved. The results, however, are contrary to previous results using *N*-acetylated heparin, which was not cleaved to any significant extent by heparinase III (Nader et al., 1990).

These results show that the linkages H_{NAc}-I and $H_{NY,6X}$-G can be degraded, when Y is either acetylated or sulfated, and when X is either sulfated or unsubstituted (Table 8). Shorter oligosaccharides are less susceptible. It remains to be determined if exhaustive cleavage leaves any tetra or hexasaccharides, with linkages that would be cleavable if presented in longer oligosaccharides, like in the case of heparinase I.

iv. Effect of 3-*O*-Sulfation

Until recently, the analysis of substrate specificity has been based on modifications of the two monosaccharides flanking a given linkage. It was found, however, that three tetrasaccharides, derived from the AT III binding region, are resistant to cleavage with heparinase I, II, and III (Rice and Linhardt, 1989; Moffat et al., 1991b; Yamada et al., 1993). NMR studies showed the generic formula to be ΔU-$H_{NAc,6X}$-G-$H_{NS,6X,3S}$, where X is either sulfated or unsubstituted (Yamada et al., 1993). Thus, it is not only the two residues flanking a bond that determines its susceptibility to cleavage. When an uronic acid has a 3-sulfated hexosamine on the reducing side, the bond on the non-reducing side of that uronic acid is resistant to cleavage. This implies that the mechanism generating specificity at the molecular level involves interaction of at least three monosaccharides with the enzyme.

v. Heparinases from other Sources

The products of heparin degradation with heparinases from *Bacteroides heparinolyticus* (Nakamura et al., 1988), *Bacillus* sp. (Bellamy, 1990) and from an unclassified bacterium (Böhmer et al., 1990) are consistent with those found for degradation with heparinase I from *F. heparinum*. However, heparinase from *Bacteroides heparinolyticus* has high relative activity on heparan sulfate (Nakamura et al., 1988), indicating that this enzyme, like heparinase II from *F. heparinum*, may cleave residues with glucuronic acid as well as residues with iduronic acid.

2. Chondroitinases

The nomenclature for the chondroitinases originated when chondroitin 4-sulfate was called

chondroitin A, dermatan sulfate was called *chondroitin B*, and chondroitin 6-sulfate was called *chondroitin C*. Hence, chondroitinase AC, for instance, cleaves chondroitin 4-sulfate and 6-sulfate but not dermatan sulfate.

Chondroitinase enzymes have been isolated and characterized from bacteria of the genera *Arthrobacter, Flavobacterium, Aeromonas, Bacillus, Bacteroides, Proteus* and an unclassified strain — possibly *Aurebacterium*. Chondroitinase activity furthermore has been detected in several other microorganisms (Salyers et al., 1977; Linhardt et al., 1986). Chondroitinase is produced in soil and intestinal bacteria; acidic polysaccharides are released to the intestinal lumen along with epithelial cells and provide intestinal bacteria with an exclusive nutrient source. Most of the strains must be induced by chondroitin sulfate or dermatan sulfate in the medium (2 to 5 mg/l), and at least two, *Bacillus* sp. and *F. heparinum*, can grow on chondroitin sulfate as the only carbon source (Linhardt et al., 1986). Characteristics of the most well studied chondroitinases are summarized in Tables 10, 11, and 12.

F. heparinum produces four different chondroitinases. Separation on a Sephacryl gel revealed the relative sizes: ABC lyase> AC lyase> C lyase > B lyase (Michelacci et al., 1987). The molecular weight of chondroitinase AC is claimed to be 70 to 75,000 (Hiyama and Okada, 1976), but a precise determination has not been published.

The chondroitinases from *Aurebacterium* sp., *P. vulgaris, Bacillus* sp. and *B. thetaiotamicron* have all been purified to single bands on native or denaturing gel electrophoresis (Linn et al., 1983; Sato et al., 1986; Takegawa et al., 1991). Under reducing (SDS) conditions the band from *P. vulgaris* ABC lyase separated into two subunits of 32 and 86 kDa (Sato et al., 1986). Two chondroitin lyases from *B. thetaiotamicron* had similar molecular weight, but there was no similarities between the tryptic digests of the two enzymes, indicating that they are not simply isomers of the same gene product (Linn et al., 1983).

Chondroitinase AC from *A. aurescens* was purified on a gel column, and then crystallized. It is the only chondroitinase, for which amino acid composition has been determined (Takegawa et al., 1991). Compared with the average composition of proteins (Creighton, 1993), it showed a normal level of arginine, and half-normal content of lysine, which is reflected in its low pI (5.5). This is surprising since a common motif for GAG-binding proteins, including heparinase I, is a high density of basic residues in the active regions (Section III.G).

a. Reaction Conditions

The optimal temperature of chondroitinase ABC from *Bacillus* sp. in a 10-min assay is as high as, 75°C, and only 20% activity is lost after 3 h (Bellamy, 1990). *A. aurescens* chondroitinase AC also has a high optimal temperature of 50°C (Hiyama and Okada, 1975b), while chondroitinase B and C from *F. heparinum* are very heat sensitive with a optimal reaction temperature around 20°C for a 4 h assay (Michelacci and Dietrich, 1976). The pH optima also varies from 5.5 for *Bacillus* sp. chondroitinase to around 8 for the *F. heparinum* chondroitinases. For chondroitinase ABC from *P. vulgaris*, the pH optima for degradation of the unsulfated chondroitin and hyaluronate were lower (6.2 and 6.8) than the optima for chondroitin and dermatan sulfate degradation (around 8) (Yamagata et al., 1968). Thus, optimal conditions tend to vary from cold and basic for chondroitinase B, C, and ABC from *F. heparinum* to hot and acidic for chondroitinases from *A. aurescens* and *Bacillus* sp.

b. Kinetic Constants

The specific activity of purified chondroitinases ranges mostly from 10 to 100 IU/mg. From *B. thetaiotamicron*, however, a purity of 433 IU/mg was obtained, while the chondroitinase ABC from *F. heparinum* had an activity of only 1.5 IU/mg (Michelacci et al., 1987) and therefore, could not be isolated properly when the *F. heparinum* chondroitinases were first characterized (Yamagata et al., 1968).

The K_m values were most often determined by reciprocal plots of initial rate versus substrate concentration expressed as mole disaccharide/liter. The K_m values for chondroitinases from *P. vulgaris, B. thetaiotamicron, A. aurescens* ,and

TABLE 10
Properties of Chondroitinase ABC from Various Sources

Source	*Proteus vulgaris*	*Flavobacterium heparinum*	*Bacillus* sp.	*Bacteroides thetaiotamicron*
Characteristics	**Fish gut Gram-negative cell associated inducible**	**Soil Gram-negative cell associated inducible**	**Soil Gram-negative secreted constitutive**	**Human colon Gram-negative cell associated inducible Type I/Type II**
Molecular properties				
Molecular weight	118,000	>70,000	53,000	104,000/108,000
pI				8.0/7.9
Activity				
T optimum (°C)	37°C	30 °C	75–80 °C	Assays were at 37 °C
pH optimum	7.9–8.3[a]		5.5–6	7.2/7.6
Specific activity (IU/mg)	4.2[1], 38[2], 117[3] 38[2] 117[3]	1.5	24.2	37/433
Turnover (s^{-1})[a]	8.3–230		21	65/765
K_m (μM disaccharide)	28			90–160 (I and II)
Relative initial rate				
Chondroitin 4-sulfate	100%	100%	100%	100% (I), 100% (II)
Chondroitin 6-sulfate	100%[2] 60%[4]	100%	90%	130% (I), 80% (II)
Dermatan sulfate	40%[2] 34%[4]	100%	15%	13% (I), 16% (II)
Chondroitin	20%[2]			200% (I), 90% (II)
Hyaluronate	2%[2] 60%[4]	0%	60%	10% (I), 30% (II)
Additives				
Inhibitors	Zn^{2+}, Heparin	Ca^{2+} (10 mM), PO_4^{3-} (10 mM) Fe^{3+}, Mn^{2+}	Fe^{3+}, Zn^{2+},Cu^{2+}, Cu^{+}, Ag^{+}, Pb^{2+} (1 mM)	Heparin I_{50} = 70–700 μM disaccharide
Compounds with no effect		Ba^{2+}, Co^{2+}	Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, β-ME, DTT, IAA, EDTA, L-cysteine	
Activators	Mg^{2+}, Mn^{2+}			
Commercially available	+			
Ref.[b]	1–5	2 and 6	7	8–10

[a] These values have been calculated from data in the original reference. The turnover number is calculated for enzymes that have been purified to homogeneity.

[b] [1]Sato et al., 1986, [2]Yamagata et al., 1968, [3]Kitamikado and Lee, 1975, [4]Thurston et al., 1975, [5]Nakada and Wolfe, 1961, [6]Michelacci et al., 1987, [7]Bellamy, 1990, [8]Guthrie et al., 1985, [9]Hwa and Salyers, 1992, [10]Linn et al., 1983.

F. heparinum (chondroitinase AC) were all between 0.03 and 0.3 mM disaccharide (Thurston et al., 1975; Hiyama and Okada, 1977; Linn et al., 1983). Chondroitinase C from *F. heparinum* had a much higher K_m (20 mM); data for chondroitinase ABC could not be fitted to a straight line, suggesting that some mechanism not following Michaelis-Menten kinetics was involved (Michelacci and Dietrich, 1976; Michelacci et al., 1987). Both of these enzymes were investigated using substrate concentrations above 0.5 mM, while the other lyases were subjected to substrate concentrations down to at least an order of magnitude less.

TABLE 11
Properties of Chondroitinase AC from Various Sources

Source	*Arthrobacter aurescens*	*Flavobacterium heparinum*	*Aurebacterium or Curtobacterium*	*Aeromonas liquefaciens*
Characteristics	**Soil Gram-positive secreted**	**Soil Gram-negative cell associated constitutive**	**Soil Gram-positive secreted inducible**	**Fish intestine Gram negative secreted**
Molecular properties				
Molecular weight	76,000	70–75,000	81–83,000	
pI	5.5			
Activity				
T optimum (°C)	50°C	40°C	37°C	stable <46°C
pH optimum	6	6.6	7.5	6.6
I optimum	0.05 *M*	<0.05 *M*	0.2 *M* (K^+)	
Specific activity (IU/mg)	72.4	11.5[1b]		
		43[2,3]	10.7	99.7
Turnover (s-1) [a]	92		15	
K_m (μ*M* disaccharide)	300–400	40–90		
Relative initial rate				
Chondroitin 4-sulfate	100%	100%	100%	100%[a]
Chondroitin 6-sulfate	110%	112%	85%	100%
Dermatan sulfate	0%	0%	0%	0%
Chondroitin	195%	100[3]–156%[4]	58%	100%
Hyaluronate	320%	20%[3], 188%[4] 107%[1]	26%	50%
Additives				
Inhibitors	Pb^{2+}, $Cu,^{2+}$, Fe^{2+}, Sn^{2+}	(Zn^{2+},Cu^{2+},Fe^{2+})[3] (Fe^{3+}, Ca^{2+},Mg^{2+},Ba^{2+})[1]	Pb^{2+},Cu^{2+},Fe^{2+}, Zn^{2+}, Hg^{2+} PCMB, IAA, NEM	Cu, Fe, Zn^{2+} Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}
Activators	Ca^{2+}, Mg^{2+}, Ba^{2+}	(Ca^{2+},Mg^{2+},Ba^{2+}, Mn^{2+})[3]	β-ME and DTT	
Heparin	No effect	Inhibitor, K_i ~ 120 μ*M*[a]		
Dermatan sulfate	Comp. inhibitor K_i = 60 m*M*	Comp. inhibitor K_i = 10 m*M*		
Commercially available	+	+		
Ref.[b]	4–7	4 and 1–3	8	9

[a] These values have been calculated from data in the original reference. The turnover number is calculated for enzymes that have been purified to homogeneity.

[b] [1]Michelacci and Dietrich, 1975; [2]Michelacci et al., 1987; [3]Yamagata et al., 1968; [4]Hiyama and Okada, 1976; [5]Hiyama and Okada, 1975a;[6]Hiyama and Okada, 1975b; [7]Hiyama and Okada, 1977; [8]Takegawa et al., 1991; [9]Kitamikado and Lee, 1975.

TABLE 12
Properties of Chondroitinase-B and C from Various Sources

Source	Chondroitinase B *Flavobacterium heparinum*	Chondroitinase C *Flavobacterium heparinum*
Characteristics	**Soil Gram-negative cell associated inducible**	**Soil Gram-negative cell associated constitutive**
Molecular properties		
Molecular weight	<70,000	<70,000, but larger than B lyase
Activity		
T optimum (°C)	20°C	20°C
pH optimum	8	7–9
I optimum	<10 m*M* NaCl	<10 m*M* NaCl
Specific activity (IU/mg)	1.3[3], 9.1[1]	
K_m (μ*M* disaccharides)		2000
Relative initial rate		
Chondroitin 4-sulfate	4%	4–20%[a]
Chondroitin 6-sulfate	0%	100%
Dermatan sulfate	100%	0%
Chondroitin		
Hyaluronate	0%	60–132%
Additives		
Inhibitors	Fe^{3+}, Ba^{2+}, Co^{2+}	Fe^{2+}, Co^{2+}, phosphate. Product inhibition by U-H_{6S} (K_i = 500 m*M*)
Compounds with no effect	Ca^{2+}	Ca^{2+}
Commercially available	+	
Ref.[b]	1–3	2 and 4

[a] These values have been calculated from data in the original reference.
[b] [1]Michelacci and Dietrich, 1975; [2]Michelacci et al., 1987; [3]Yamagata et al., 1968; [4]Michelacci and Dietrich, 1976.

Of the ABC chondroitinases, only the one from *F. heparinum* is fully active against dermatan sulfate. The other three have dermatan sulfate activities (initial reaction rate) ranging from 13 to 40% of their chondroitin 4-sulfate activity. Dermatan sulfate from pig skin and intestine contain around 20% glucuronic acid (Maimone and Tollefsen, 1990; Linhardt et al., 1991); thus, it is possible that these chondroitinases are specific for glucuronic acid and show lower initial rates with dermatan sulfate as substrate due to the lower glucuronic acid content of dermatan sulfate.

None of the chondroitinases showed any activity toward heparin or heparan sulfate, which are entirely 1,4 linked. This indicates, that the 1,3 linkage of chondroitin sulfate is a very strong determinant for enzymatic activity, even though it is the 1,4 bond of chondroitin sulfates that is cleaved by chondroitinase.

The relative activities with the unsulfated substrates, chondroitin and hyaluronic acid as substrates, vary considerably. It is higher for ABC chondroitinases with low dermatan sulfate activity, and for the AC chondroitinases, than for the ABC chondroitinases with high dermatan sulfate activity, and for chondroitinase B. The chondroitinase ABC from *B. thetaiotamicron* has high activity against the unsulfated chondroitin, but the K_m for this substrate is one order of magnitude higher than K_m for chondroitin sulfate, indicating that the lack of sulfates in chondroitin makes it a poorer substrate (Linn et al., 1983). These observations indicate that the chondroitinases in addition to degrading chondroitin sulfates are active,

either against the unsulfated substrates or against dermatan sulfate, but not against both.

Four of the chondroitinases (AC from *A. aurescens* and *F. heparinum* and ABC from *B. thetaiotamicron* and *P. vulgaris*) were inhibited by other GAGs such as heparin and dermatan sulfate (Nakada and Wolfe, 1961; Linn et al., 1983). It is striking that the dissociation constants for the inhibitors (K_i) are the same, or even 1 to 3 orders of magnitude smaller than K_m. Thus, the inhibitors bind but they cannot be cleaved, suggesting that the enzyme specificity is determined by the mechanism of cleavage rather than by the mechanism of binding. It is possible that the strong inhibitor binding is a non-specific polyelectrolyte phenomena due to the higher charge density of heparin. Two observations emphasize that the highly negatively charged heparin inhibits by binding to the enzyme: heparin binds with highest affinity to the *B. thetaiotamicron* chondroitinase, which has a rather high pI of 8. It does not have any inhibitory effect on *A. aurescens* chondroitinase AC lyase, which has the lowest pI of 5.5 and consequently an overall negative charge at reaction conditions (Hiyama and Okada, 1977).

Chondroitinase AC from *Aurebacterium* was inhibited by the sulfhydryl reactants PCMB, IAA and NEM, but enhanced by β-mercaptoethanol and dithiothreitol (Takegawa et al., 1991). This suggests that a reduced cysteine is important for the conformation of active site or for the mechanism of cleavage.

c. Substrate Specificities for F. heparinum Chondroitinases

i. Chondroitinase ABC

Chondroitinase ABC causes the weight average molecular weight of the substrate to decrease slowly, and no oligosaccharide intermediates were produced when radio labeled products were monitored by gel electrophoresis. Chemical modification of the substrate termini indicated, that this enzyme acts exclusively on the reducing end of the GAG (Michelacci et al., 1987). These findings indicate an exolytic activity. The chondroitinase ABC could not be modeled by Michaelis-Menten kinetics since it is inhibited by its product ($U\text{-}H_{NAc,4S}$) at 2.3 m*M* and possibly also by excess substrate (Michelacci et al., 1987).

ii. Chondroitinase AC

On the other hand, chondroitinase AC produces an intermediary hexasaccharide, as well as a tetrasaccharide that from paper chromatography analysis seems to reach a steady level from 1 to 4 h into the reaction (Michelacci et al., 1987) and then possibly become degraded later (Michelacci and Dietrich, 1975). The structure of this tetrasaccharide has not been resolved. The molecular weight of the substrate drops significantly at the onset of reaction. This product formation indicates a predominately endolytic mode of action.

iii. Chondroitinase B

Chondroitinase B degradation of dermatan sulfate from various sources was analyzed with ion exchange HPLC and yielded as main products: $U\text{-}H_{4S}$, $U\text{-}H_{4S,6S}$, $U_{2S}\text{-}H_{6S,}$ and $U_{2S}\text{-}H_{4S}$ (Murata and Yokoyama, 1987; Linhardt et al., 1991). These disaccharides accounted for 50 to 65% of the 232-nm absorbance while the remaining 35 to 50% absorbance most likely originated from unidentified oligosaccharides (Linhardt et al., 1991). However, degradation of oversulfated dermatan sulfate isomers additionally yielded the trisulfated disaccharide, $U_{2S}\text{-}H_{4S,6S}$ (Murata and Yokoyama, 1987). Thus, assuming there was no contaminating enzyme activities, chondroitinase B can cleave bonds that contain 2-sulfate, 6-sulfate or both in addition to the normal 4-sulfate found in most dermatan sulfate linkages.

Degradation of pig skin dermatan sulfate for 24 h with chondroitinase B yielded 36 weight % disaccharide $U\text{-}H_{4S}$, 23% tetrasaccharides, 20% hexasaccharides and 31% of an oligosaccharide, as determined by paper chromatography (Michelacci and Dietrich, 1975). The tetra- and hexasaccharides could be further degraded by 2.5 times higher concentrations of B lyase, but they were resistant to chondroitinase AC. The oligosaccharide, on the other hand, was degradable by chondroitinase AC but completely resistant to further chondroitinase B degradation. The oligosaccharide was monodis-

perse with molecular weight of 3000 and it had the same average degree of sulfation and *N*-acetylation as the other products and the substrate (Michelacci and Dietrich, 1975). It is surprising that dermatan sulfate is resistant to chondroitinase AC but that the dermatan sulfate-derived oligosaccharide (31% by weight) could be degraded by this enzyme. This suggests that the local sequence of residues is not the primary determinant for susceptibility to chondroitinase, and that other factors, including distant residues of the chain, are more important in this case. Perhaps the overall conformation of the isolated oligosaccharide is different from its conformation as part of a dermatan sulfate chain. Thus, the conformation of substrate may be a discriminating factor for its chondroitinase AC susceptibility.

iv. Chondroitinase C

Finally, chondroitinase C had a low but detectable activity against chondroitin 4-sulfate in addition to its primary activity against chondroitin 6-sulfate. Paper chromatography of the chondroitin 6-sulfate degradation products showed, a large spot corresponding to the 6-sulfated disaccharide, and some oligosaccharides left (presumably containing 4-sulfated residues). The products from chondroitin 4-sulfate degradation were small amounts of 6-sulfated disaccharide and large amounts of oligosaccharides. In both cases no 4-sulfated disaccharides could be detected. This confirms, that the enzyme is indeed specific for 6-sulfated linkages, and that the crossreactivity with chondroitin 4-sulfate is due to the presence of copolymers of chondroitin 4-sulfate and chondroitin 6-sulfate residues (Michelacci and Dietrich, 1976).

d. Substrate Specificity for P. vulgaris Chondroitinase ABC

There are contradictory results in the literature regarding the action pattern of chondroitinase ABC from *P. vulgaris*. Using size-exclusion chromatography, Yamagata et al. found that the disaccharide peak rose immediately (within 10 min), and that little or no oligosaccharide intermediates were formed from digestion of chondroitin 4-sulfate (Yamagata et al., 1968), whereas Sanderson et al. observed equal amounts of di-, tetra-, and hexa-saccharides after 1 to 3 h and pure disaccharide after 31 h in a similar experiment (Sanderson et al., 1989). Jandik et al. found that the change of viscosity during the course of reaction showed a predominately endolytic mechanism with chondroitin 6-sulfate or dermatan sulfate as substrates and an exolytic mechanism with chondroitin 4-sulfate (Jandik et al., 1994). Thus, it is unclear if the enzymatic action pattern is exolytic or endolytic.

The final degradation products of dermatan sulfate from bovine and porcine intestines were determined by SAX-HPLC (Linhardt et al., 1991). The major disaccharide was U-H_{4S} (~60 mol %) but also U-H_{6S}, U_{2S}-H_{4S}, U_{2S}-H_{6S}, U-$H_{4S,6S}$ and U_{2S}-H were formed (~20 mol %). This indicates a rather broad specificity in terms of sulfation as well as type of uronic acid.

e. Substrate Specificity for A. aurescens Chondroitinase AC

Hiyama and Okada published a series of papers clarifying the mechanisms of action of AC chondroitinases from *A. aurescens* and *F. heparinum* (Hiyama and Okada, 1975b; Hiyama and Okada, 1975a; Hiyama and Okada, 1976; Hiyama and Okada, 1977). They found that *A. aurescens* chondroitinase AC cleaves more linkages and produces disaccharides from the start of the reaction, compared with *F. heparinum* chondroitinase AC, which does not produce disaccharides until late in the reaction (Hiyama and Okada, 1976). Recently, these results were supported by GPC and gradient PAGE analysis which showed that chondroitinase AC from *A. aurescens* does not produce any oligosaccharides of intermediary length during the course of reaction (Jandik et al., 1994). Also, the viscosity of the substrate solution drops faster when chondroitin 4-sulfate or chondroitin 6-sulfate is degraded with *A. aurescens* chondroitinase AC than when they are degraded with *F. heparinum* chondroitinase AC (Jandik et al., 1994). These results indicate that *A. aurescens* chondroitinase acts predomi-

nately exolytically, while *F. heparinum* chondroitinase acts randomly endolytically.

Dermatan sulfate can be degraded to a very low extent by chondroitinase AC. The products account for approximately 7% of the disaccharide linkages in dermatan sulfate, reflecting the content of glucuronic acid, and indicating this enzyme cleaves only linkages with glucuronic acid (Linhardt et al., 1991; Gu et al., 1993b). There has been no identification of the disaccharide products from degradation of chondroitin sulfates, the main substrates of chondroitinase AC, indicating that the substrate was not contaminated in these studies.

In general, the chondroitinases show strong preference for linkages containing either glucuronic acid or iduronic acid. The activity against dermatan sulfate on one hand and the unsulfated chondroitin and hyaluronic acid on the other seem to be mutually exclusive. This suggests that two different catalytic mechanisms are utilized. In one case chondroitinase interacts predominately with the sulfate groups, thus crossreacting with dermatan sulfate that has similar sulfation as chondroitin 4-sulfate. The other involves interaction between enzyme and the uronic acid, leading to crossreaction with the unsulfated polysaccharides but not with dermatan sulfate. Several chondroitinases are inhibited by heparin and dermatan sulfate, and the inhibitory constants are surprisingly low compared with K_m for the substrate, which means that the enzyme has a high affinity for the inhibitor. Thus, chondroitinases show high specificity with respect to catalysis but low specificity with respect to binding.

3. Hyaluronidases

Hyaluronidase has been isolated from bacterial as well as animal sources and its activity has been detected in a streptococcus bacteriophage. The bacterial sources include: *Propionibacterium, Peptostreptococcus, Staphylococcus, Streptococcus,* and *Streptomyces* genera (Linhardt et al., 1986). Hyaluronidase also can be purified from the venom of the honey bee *Apis mellifera* (Kemeney et al., 1984). The properties of these enzymes are summarized in Table 13.

The enzyme activity is generally not a strong function of pH, they show a broad, slightly acidic optimum (Vesterberg, 1968; Tam and Chan, 1985; Hamai et al., 1989). The *S. dysgalactiae* and *Peptostreptococcus* sp. derived hyaluronidases have turnover numbers (mole products/mol enzyme/second) that are orders of magnitude higher than any other polysaccharide lyase described in this review. These two observations suggest some fundamental difference in the mechanism of cleavage.

The *Peptostreptococcus* sp. hyaluronidase has low activity toward chondroitin 4-sulfate and chondroitin 6-sulfate and it was completely inactive toward dermatan sulfate and heparin (Tam and Chan, 1985). The K_m value based on disaccharides is 0.3 m*M* for hyaluronic acid, but higher (3 to 6 m*M*) for the chondroitin sulfates (Tam and Chan, 1985). Hyaluronic acid is completely degraded to disaccharides after 24 h, as determined by gel chromatography. This is different from the testicular hyaluronate hydrolase, which produces multiple intermediates and leaves tetra- and hexasaccharides after 24 h degradation (Yamagata et al., 1968; Tam and Chan, 1985).

Two isomeric forms of the hyaluronidase from *Staphylococcus aureus* were found by isoelectric focusing, one at pI 7.4 (A) the other at 7.9 (B). During cultivation, and after storage, form A could be converted to B, indicating they are the same gene product modified differently (Vesterberg, 1968). This hyaluronidase was inhibited by 10 m*M* iodoacetic acid (IAA), but not influenced by cysteine, NEM, or EDTA. The inhibition by IAA indicates cysteine residues are involved in the mechanism. It is surprising that EDTA, which chelates calcium strongly, does not affect the reaction because the structure of hyaluronic acid is highly influenced by calcium (Section III.E).

Greiling and co-workers studied hyaluronidase from a *Streptococcus* Group A bacterium and found that photo oxidation eliminated enzymatic activity and destroyed all histidine residues as well as some lysine and methionine (Greiling et al., 1975). Incubation with histidine-specific reagents (TLCK and TPCK) reduced activity to 50%. The authors suggested a model in which a histidine residue acts as a general acid-base catalyst, thereby extracting a proton from the uronic acid, and eliminating the glycosidic bond.

TABLE 13
Properties of Hyaluronic Acid Lyases from Various Bacterial and Animal Sources

Source	*Peptostreptococcus* sp. Periodontal pocket	*Staphylococcus aureus*	*Streptococcus dysgalactiae*	*Apis Mellifera* (honey bee) venom
Characteristics	**Secreted**	**Secreted constitutive**	**Secreted inducible**	
Molecular properties				
Molecular weight	160,000	84,000	117–125,000	35–40,000
pI		7.4/7.9 (two isomers)		9.0
Amino acid composition		> 9.5% lysine > 6.6% histidine		5.7 % lysine 7.2 % arginine
Activity				
T optimum	46°C (55 min)		37°C (10 min)	
pH optimum	6.6–7.4	4.8–6.0	5.8–6.6	
I optimum		0.15 *M* (phosphate)		
Specific activity (IU/mg)	600,000	15	1480	37[a]
Turnover (s^{-1})[a]	1.6 10^6	21	3000	23
K_m (μM disaccharides)	Hyaluronate: 300 Chon.-4S: 3000 Chon.-6S: 6000		Polysaccharide: 50 Tetrasacch.: 920	
Relative initial rate	Hyaluronate: 100% Chon.-4S 10% Chon.-6S: 5% (pH 8)		Hyaluronate: 100% Chondroitin: <5%[a] Chon.-4S : 0%	
Additives				
Inhibitors		IAA (10 m*M*)	Fe^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}, Zn^{2+}	
Compounds with no effect		Cysteine, EDTA , and NEM	EDTA, NaCl (<0.25 *M*)	
Comm. available			+	
Ref.	Tam and Chan, 1985	Vesterberg, 1968; Rautela and Abramson, 1973	Hamai et al., 1989	Kemeney et al., 1984

[a] These values have either been calculated from data or estimated from graphs in the original reference. The turnover number is calculated for enzymes that have been purified to homogeneity.

B. Hydrolases

The natural metabolism of GAGs in animals is carried out by hydrolases. Generally the GAGs are degraded in a two step procedure (Hopwood, 1989; Kresse, 1989; Roden et al., 1989) (Table 14). First, the proteoglycans are internalized in endosomes where an initial depolymerization of the GAG chains takes place. This step is mainly endolytic and yields oligosaccharides. Further degradation is carried out after fusion with lysosomes, a more acidic compartment of the cell, where desulfation and exolytic depolymerization to monosaccharides take place. The sulfatase enzymes have been reviewed by Hopwood (Hopwood, 1989) and are not included here. In addition to the animal sources, a number of plants and microorganisms also produce GAG hydrolases.

Unlike the unsaturated products of eliminative cleavage, the degradation products from hydrolysis of GAGs do not absorb UV light and radioactive labels and chemical assays for uronic acid must be used to quantitate the products. This introduces ambiguity in the definition of activity units that are often impossible to correlate to moles of cleaved bonds.

1. Heparin Hydrolases

Endo-β-D-glucuronidases called heparanases have been found in platelets, T- and B-lymphocytes, macrophages, malignant tumor cells, fibroblasts and rodent ovarian cells (Nakajima et al., 1984; Savion et al., 1987; Rosenberg, 1989; Godder et al., 1991; Laskov et al., 1991; Matzner et al., 1992; Bame, 1993). Heparanases that are either constitutively expressed and secreted from tumor cells or stored intracellularly in circulating cells from which they can be released on stimulation have been studied most extensively (Vlodavsky et al., 1992). The presumed function is to degrade the extracellular matrix to facilitate extravasation from capillaries or tumor growth expansion. These heparanases are generally more active towards heparan sulfate than toward heparin. They cleave the (β1,4) linkage at the nonreducing side of glucosamine residues. Both platelet and tumor (melanoma)-derived heparanases are specific for linkages with glucuronic acid (Oosta et al., 1982; Nakajima et al., 1984; Nakajima et al., 1986), but they have subtle differences in specificity and product profile.

Platelet heparanase is a single chain molecule with molecular weight of 134 kDa and a broad pH

TABLE 14
Depolymerizing Enzymes of Animal Metabolism of GAGs

Degradation step	Enzyme	Substrates
Initial	Heparanase	Heparin, HS
	Hyaluronidase	Hyaluronic acid, chondroitin sulfate, dermatan sulfate
	Endo-galactosidase[a]	Keratan sulfate
Subsequent	β-glucuronidase	HS, chondroitin sulfate
	α-L-iduronidase	Heparin, dermatan sulfate
	β-galactosidase	Keratan sulfate
	β-*N*-acetylhexosaminidase	Hyaluronate, keratan sulfate, chondroitin sulfate, dermatan sulfate
	α-*N*-acetylglucosaminidase	Heparin, HS

[a] Presumably, an endo-galactosidase initially depolymerizes keratan sulfate, but its general importance in animals has not been definitively proven.

optimum for activity of 5.5 to 7.5 (Oosta et al., 1982; Rosenberg, 1989). The degradation of both heparan sulfate and heparin yields hexasaccharides and larger oligosaccharides as final products (Wasteson and Westermark, 1976; Wasteson et al., 1977). Inspection of the degradation products indicated that only linkages with glucuronic acid are cleaved (Oosta et al., 1982). There does not seem to be any restrictions on the residues to the non-reducing end of the cleaved linkage. It can be either *N*-acetylated, *N*-sulfated, or 2-sulfated on IdoUA (Table 8). Thunberg and co-workers incubated platelet heparanase with an AT III binding octasaccharide and found that even this small saccharide can be cleaved, and that 3-sulfation of a glucosamine on the reducing side of a bond does not impair its susceptibility (Thunberg et al., 1982).

Tumor heparanase was investigated using melanoma cell extracts from mice and humans (Nakajima et al., 1984; Nakajima et al., 1986). There was no activity toward keratan sulfate, hyaluronic acid, or chondroitin 4-sulfate and only 10 to 20% of chondroitin 6-sulfate and heparin was mobilized from the original spot in a gel chromatogram. The pH optimum was slightly more acidic than for the platelet heparanase, but this could be an effect from the residual cell extract. Heparin was a potent inhibitor of the activity of heparanase toward heparan sulfate.

The melanoma derived heparanase has increased activity toward an *N*-acetylated heparin, and reduced activity toward an *O*-desulfated heparan sulfate (Nakajima et al., 1986). A mastocytoma-derived heparanase requires *O*-sulfation to be fully active, probably at the 2-position of iduronic acid (Thunberg et al., 1982). These enzymes cannot degrade an octasaccharide derived from the AT III binding region, and they do not destroy the anticoagulant activity of heparin (Thunberg et al., 1982). This indicates that 3-*O*-sulfation of glucosamine inhibits reaction. For heparanases from mouse and human melanomas, the degradation products are quite large, and the profiles are identical. Intermediary products of 10 to 20 kDa were reduced to 5 to 6 kDa after 6 h incubation (Nakajima et al., 1984; Nakajima et al., 1986). This degradation pattern also was found for heparanase from rat ovarian cells (Hopwood, 1989; Yanagishita, 1989).

Thus, the platelet heparanase has broad specificity and leaves small oligosaccharides, while the tumor heparanases are specific for linkages with *N*-acetylation and 2-sulfation of adjacent iduronic acids, and their products are larger oligosaccharides (Table 8). This may reflect the difference between the cellular origin of these enzymes: platelets and other circulatory cells release heparanase in response to certain stimuli, while tumor heparanases are constitutively secreted in pathological situations.

2. Keratanases

Inasmuch as keratan sulfate does not have any uronic acid, the eliminase reaction is not possible, and hydrolases are the only enzymes depolymerizing this GAG. The keratanases can cleave either at the β1,3 linkage at the reducing side of the hexosamine, like the lyases, or at the β1,4 linkage at the non-reducing side of the hexosamine (Table 8).

An endo-β-D-galactosidase that can depolymerize keratan sulfate has been detected in the kidney, but it has not been established if this enzyme has a general metabolic function (Kresse, 1989). Three types of microbial keratanases have been purified (Table 15). *Bacteroides fragilis* and *Flavobacterium keratolyticus* produce the enzyme constitutively, while *Escherichia freundii*, *Pseudomonas* sp. and an unclassified bacterium must be induced by keratan sulfate. A review by Nakazawa et al. (Nakazawa et al., 1989) incorporates many non-published observations on the properties of these enzymes.

a. Endo-β-galactosidase and Keratanase I (β1-4)

The endo-β-galactosidase and keratanase I both cleave β-1,4 linkages, but differ in their specificity for sulfation pattern. Endo-β-galactosidase from three different bacterial sources seem to have identical pH optimum and specificity, as indicated in Table 15 (Scudder et al., 1986; Nakazawa et al., 1989).

On degradation of bovine keratan sulfate with keratanase I or endo-β-galactosidase, gel-chromatography showed that 75 to 80% of the galac-

TABLE 15
Bacterial Keratanases and their Substrate Specificities

	Endo-β-galactosidase	Keratanase I	Keratanase II
Source	*E. freundii*, *F. keratolyticus*,	*Pseudomonas* sp.	Unclassified bacterium
Commercially available		+	
pH optimum	5.8	7.4	
Specificity	$H_{NAc,6X}$-Gal-$H_{2Y,6X}$ ↑	$H_{NAc,6X}$-Gal-$H_{NAc,6S}$ ↑ or $H_{NAc,6S}$-Gal-$H_{NAc,6X}$ ↑	$H_{NAc,6S}$-Gal_{6X}-$H_{NAc,6S}$ ↑

Note: X is unsubstituted or sulfated; Y is unsubstituted or *N*-acetylated.

tose residues were found in smaller oligosaccharides (n <10) (Kresse, 1989; Nakazawa et al., 1989). The main degradation products were a monosulfated disaccharide (H_{6S}-Gal), and a trisulfated tetrasaccharide (H_{6S}-Gal_{6S}-H_{6S}-Gal) identified by negative ion FAB mass spectroscopy. Shark keratan sulfate, which has a higher degree of sulfation, was less susceptible but the degradation increased when this keratan sulfate was desulfated chemically (Nakazawa et al., 1989). These observations indicate that sulfation of galactose impairs the cleavage with both of these enzymes.

Keratanase I reached optimal activity when the degree of sulfation was around one per disaccharide. Endo-β-galactosidase, on the other hand, became even more active when the substrate was desulfated to less than one sulfate per disaccharide and produced an unsulfated disaccharide from the desulfated keratan sulfate (Nakazawa et al., 1989). Furthermore, fractions of the oligosaccharides from keratanase I degradation could be degraded by endo-β-galactosidase. This yielded two different disaccharides: H_{6S}-Gal and H-Gal. Thus, the difference between the two enzymes is that keratanase I requires sulfation of one of the glucosamine residues adjacent to the galactose of the cleaved linkage. From these studies, however, it cannot be inferred whether it is the glucosamine on the reducing side or the one on the non-reducing side which must be sulfated, or if it can be either.

Endo-β-galactosidase has a broad specificity. Unlike keratanase I, it also degrades various glycosphingolipids with linkages like Glc_{NAc}-Gal-Glc, and the unsulfated Gal-Glc_{NAc} linkages of erythroglycan (Nakagawa et al., 1980). However, a linkage becomes resistant to cleavage by isomerization of the glucosamine to galactosamine on the non-reducing side. Thus, the sequence Gal_{NAc}-Gal-Glc_{NAc} is resistant to cleavage.

b. Keratanase II

Keratanase II cleaves β1,3 linkages on the non-reducing side of both 6-sulfated and non-sulfated galactose residues. The highly sulfated shark cartilage keratan sulfate could be degraded by this enzyme to di- and tetrasaccharides comprising 80% of the galactose residues from the original substrate (Nakazawa et al., 1989). Bovine corneal keratan sulfate yielded two distinct disaccharides, one monosulfated at the glucosamine residue and one disulfated (Nakazawa et al., 1989).

3. Hyaluronic Acid Hydrolases

Two classes of hyaluronic acid hydrolases have been isolated. Type I, also called testicular hyaluronidase (EC 3.2.1.35), cleave on the non-reducing and type II, leech hyaluronidase (EC 3.2.1.36), on the reducing side of glucuronic acid (Table 8). The testicular hyaluronidase also is active towards chondroitin and dermatan sulfates, and it has a transglycosylation activity that can transfer a disaccharide residue between two oligomers (Roden et al., 1989).

Both enzymes yield a series of oligosaccharides from 1 to 23 disaccharide units (Fransson, 1985; Fransson et al., 1990) after short reaction times of 2 min. Testicular hyaluronidase forms many oligosaccharide intermediates and no disaccharides early in the reaction. The end product after exhaustive degradation is a mixture oligosaccharides with tetra- and hexasaccharides being the main products (Yamagata et al., 1968; Tam and Chan, 1985). This indicates a random endolytic activity with low affinity for small oligosaccharides.

Studies with radiolabeled oligosaccharides revealed that the catalytic site of testicular hyaluronidase binds a decasaccharide and cleaves between residue 4 and 5 from the non-reducing end (Highsmith et al., 1975; Matheson and McCleary, 1985). Also, when the hexasaccharide $(GlcUA\text{-}GlcNAc)_3$ was treated with testicular hyaluronidase, the products included tetra and octasaccharides but no disaccharides (Highsmith et al., 1975). This suggests that transglycosidase activity can affect the product profile significantly.

4. Exo-glycosidases

The exo-glycosidases act on the non-reducing end of the oligosaccharide substrate. Five enzymes belong to this category: β-glucuronidase (EC 3.2.1.31), α-L-iduronidase (EC 3.2.1.76), β-galactosidase (EC 3.2.1.23), β-*N*-acetylhexosaminidase (EC 3.2.1.52), and α-*N*-acetylglucosaminidase (EC 3.2.1.50). Their pH optima ranges from 4 to 6, reflecting that they are adapted to work in an acidic intracellular environment (Kresse and Glossl, 1987; Hopwood, 1989). The specificities of the exo-glycosidases are illustrated in Table 8.

The two uronidases are specific for the anomeric conformation of the link they cleave (α or β), and it is remarkable that they do not discriminate between a 1,4 link as in heparan sulfate or a 1,3 link as in dermatan sulfate. These are the only two enzymes described in this review, which can cleave members of both the heparin/heparan sulfate and chondroitin/dermatan sulfate families of GAGs. Also the strict exolytic activity and specificity for de-sulfated terminal residues at the non-reducing end distinguish them from other GAG depolymerizing enzymes. It is likely that conformational freedom of the terminal residue makes it possible for both 1,4 linked and 1,3 linked uronic acids to fit in the active site of the enzyme. More restricted structures of the internal uronic acids may prevent endo-lyases and endo-hydrolases from crossreacting with both heparin-like and chondroitin sulfate-like polysaccharides.

C. Main Characteristics of GAG-Depolymerizing Enzymes

The literature on GAG depolymerizing enzymes has focused on the specificity of enzymatic activity on heterogeneous substrates in addition to characterization of molecular properties and optimal reaction conditions.

There has been much interest in the electrostatic properties of lyases because the GAG substrates have a very high negative charge density. Heparinases usually have pI around 9, but heparinase I from *Bacillus* sp. and chondroitinase AC from *A. aurescens* have low pI around 6. This demonstrates that the overall charge of the enzyme not necessarily has to complement the charge of the substrate. For heparinase I the high pI often is explained by the high lysine content of 11% (Lohse and Linhardt, 1992). However, heparinase II and III have the same lysine and arginine content as *Bacillus* sp. heparinase and yet 3 units higher pI. Thus, one most be cautious in interpreting results of chemical amino acid analysis, which does not provide the content of negatively charged amino acids. This information can only be obtained from gene sequencing.

The reaction kinetics can be complicated. Even though the data can be fitted to a straight line in a Lineweaver-Burk plot of a narrow concentration range, the kinetics may change considerably with larger changes in substrate concentration, reflected in the large discrepancies in K_m found for heparinase I (Yang et al., 1985; Rice and Linhardt, 1989; Lohse and Linhardt, 1992). The kinetics of hyaluronidases differ in two aspects from the general picture. They are not as sensitive to pH changes as other lyases and although most lyases have turnover numbers around 15 to 200 s^{-1}, two hyaluronidases have values several orders

of magnitude higher (Tam and Chan, 1985; Hamai et al., 1989).

The mechanisms of catalysis have mainly been elucidated through product analysis. Separation techniques for analysis have evolved from paper chromatography, gel chromatography, and gel-electrophoresis to HPLC and CZE. Similarly, the methods of identifying the various peaks have evolved from chemical analyses that had poor recovery for sulfated GAGs, to 1D-NMR of disaccharides and 2D-NMR of di-, tetra-, and hexasaccharides (Linhardt et al., 1992b). Recently, mass spectroscopy has shown promise for identification of higher oligosaccharides (Juhasz and Biemann, 1994).

Only heparinase I from *F. heparinum* has been studied by all of these techniques. Around ten peaks in the degradation spectrum have been identified and quantified, revealing hitherto unknown degradation products (Linhardt et al., 1992a). Products from cleavage of heparan sulfates with heparinase II and III have been separated by SAX-HPLC, and the disaccharide products characterized (Desai et al., 1993b). However, the amount and composition of intermediate and final oligosaccharides have not been elucidated. The information on heparinases from other strains is sparse, but the large difference in pI between *F. heparinum* and *Bacillus* sp. heparinases suggests that also the mechanism may be different.

There seem to be two classes of chondroitinases, with two different catalytic mechanisms, interacting predominately with either the sulfate groups, or the uronic acid. Catalysis is specific, such that bonds with different sulfate groups or uronic acids are not cleaved, but binding seems to be less specific because inhibitors that are not cleaved themselves bind with high affinity (Linn et al., 1983).

The substrate specificity of the enzymes has generally been based on the modification patterns on the two monosaccharides closest to a particular linkage. The susceptible linkages are summarized in Table 8. Specificity is primarily determined by the sugars and linkages of the repeating unit, while there are some cases where specific sulfation pattern is governing. Recently, however, it was realized that the sulfation pattern of residues further away from a linkage also could be determinants for its susceptibility (Yamada et al., 1993). It was shown that when an uronic acid has a 3-sulfated glucosamine on the reducing side, the bond on the non-reducing side of that uronic acid is resistant to cleavage by heparinase I, II, and III. This implies that the mechanism generating specificity at the molecular level involves interaction of at least three monosaccharides with the enzyme.

Heparinase II and chondroitinase ABC are remarkable enzymes since they cleave linkages with iduronic acid as well as linkages with its isomer, glucuronic acid. Also, β-glucuronidase and α-L-iduronidase can cleave both 1,4 and 1,3 linkages between the hexosamine and the uronic acid. It would be interesting to elucidate the molecular design of these enzymes, which cleave such diverse substrates, whereas all other GAG degrading enzymes are specific for one uronic acid and one type of linkage.

D. Enzyme-Substrate Interactions

The discussion in the previous sections suggest modeling the enzyme-substrate interaction in binding and catalysis to elucidate the mechanism of GAG degradation. The first studies of such interaction at a molecular level between a GAG and its degradative enzyme were made possible by cloning and sequencing heparinase I from *F. heparinum* (Sasisekharan et al., 1993).

1. Molecular Model for Heparin-Heparinase Binding and Catalysis

Cloning and expression of heparinase I has led to improved understanding of the mechanisms of heparinase binding and activity. The heparinase I gene from *F. heparinum* was cloned and sequenced, and the protein product expressed and purified from *E. coli* (Sasisekharan et al., 1993; Ernst et al., 1995). The region of amino acids 196 to 213 contains two sequential consensus sequences for heparin binding (Sasisekharan, 1991). Cardin and Weintraub originally suggested the two consensus sequences for heparin binding: X-B-B-X-B-X and X-B-B-B-X-X-B-X, where X is any hydrophilic amino acid and B is a basic amino acid (Cardin and Weintraub, 1989). From cyanogen bromide and tryptic digests of heparinase I, peptides were isolated that bound specifically

to heparin and not to chondroitin sulfate. These peptides were sequenced and found to include amino acids 196 to 221, consistent with the consensus sequence hypothesis (Sasisekharan et al., 1995a). A peptide (HBP1) identical to the consensus sequences (196-213) was synthesized (Sasisekharan et al., 1995a), and shown to bind to heparin dodecasaccharides.

Sulfhydryl derivatization with *p*-chloromercuribenzoate (PCMB) and iodoacetic acid (IAA) impaired the activity of the enzyme but not the heparin-binding properties (Sasisekharan et al., 1995a; Sasisekharan et al., 1995b). Thus, at least one cysteine residue is essential for the catalytic activity. Subsequent tryptic digestion revealed that the derivatized peptide contained amino acids 133 to 167 and that cys 135 therefore is part of a catalytic site (Sasisekharan, 1991). It was further confirmed that the catalytic site is charge dependent, since the neutral reagents *N*-ethylmaleimide (NEM) and iodoacetamide were not inhibitory (Sasisekharan et al., 1995b). Substituting cysteine 135 with alanine completely abolished activity, while some activity was retained after substitution with serine, confirming the intricate involvement of this residue in the catalytic site as a possible nucleophile (Sasisekharan et al., 1995b). Eliminative cleavage is believed to involve proton abstraction from C5 of iduronic acid (Linhardt et al., 1986). The thiol group of cysteine normally has a pKa of 8.35 (Fersht, 1985), indicating that it will be fully protonated at the pH optimum for heparinase I of 7. It is possible, however, that a positively charged environment from nearby lysines or arginines will tend to keep the thiol group negatively charged (i.e., lowering its pKa) so that it can act as a base for proton abstraction. This theory is supported by kinetic studies which showed a comparable rate constant for inactivation of cysteine 135 by IAA at pH 6.5 and 8.5, indicating that the effective pKa of cysteine 135 is around or below 6.5 (Sasisekharan et al., 1995b). Further, a heparinase I mutant with alanine replacing histidine 203 from the heparin binding region was inactive, indicating that this residue is close to the scissile bond during catalysis (Sasisekharan et al., 1995a).

These experiments have shown that heparinase I has a binding site homologous to those of several mammalian heparin binding proteins, and a catalytically active cysteine 135. These two functional sites in the primary sequence appear to be brought in close proximity in the folded protein, such that the basic heparin binding region provides a positive microenvironment for the catalytically active cysteine 135, in addition to complementing the charge of the substrate.

2. Three-Dimensional Structure of Pectate Lyase

The structures of GAG degrading enzymes have not been solved. Recently, however, the crystal structure of the pelC pectate lyase from *E. chrysanthemi* expressed in *E. coli* was solved (Yoder et al., 1993a). The structure revealed a novel super secondary motif, a "β-helix," which is a coil of 22 residues per turn, and 0.22 Å rise per residue (Cohen, 1993; Yoder et al., 1993b) (Figure 7). In this structure, the flanks of the spiral can interact as parallel β-strands through hydrogen bonds. The interior of the spiral is occupied by side chains, among which a series of asparagines in six consecutive turns form a ladder. Calcium is necessary for pectate lyase activity and the crystal structure provides a possible binding site for calcium at the exterior of the helix (Yoder et al., 1993a; Yoder et al., 1993b). This site is in a groove on the surface which can accommodate a dodecagalacturonate, as suggested by computer docking of enzyme and substrate. The immediate surroundings of the calcium site, consisting of the charged groups asp-170, lys-172 and arg-218, are strongly conserved in a series of pectate lyases (Hinton et al., 1989) making it a very likely candidate for a catalytic site.

This is the first structure of a polysaccharide lyase; the structures of pectate lyase E from *E. chrysanthemi* (Yoder et al., 1993b) and pectate lyase from *Bacillus subtilis* (Pickersgill et al., 1994) were subsequently solved and found to be similar to the β-helix structure of pectate lyase C. It is inviting to speculate that this β-helix motif may be shared by other GAG lyases, thus serving as a model for these. However, the sequence homology between pectate lyase and heparinase I is too low for direct homology modeling. The involvement of calcium in the putative catalytic site suggests that this cation plays a crucial role not only for the secondary structure of pectate

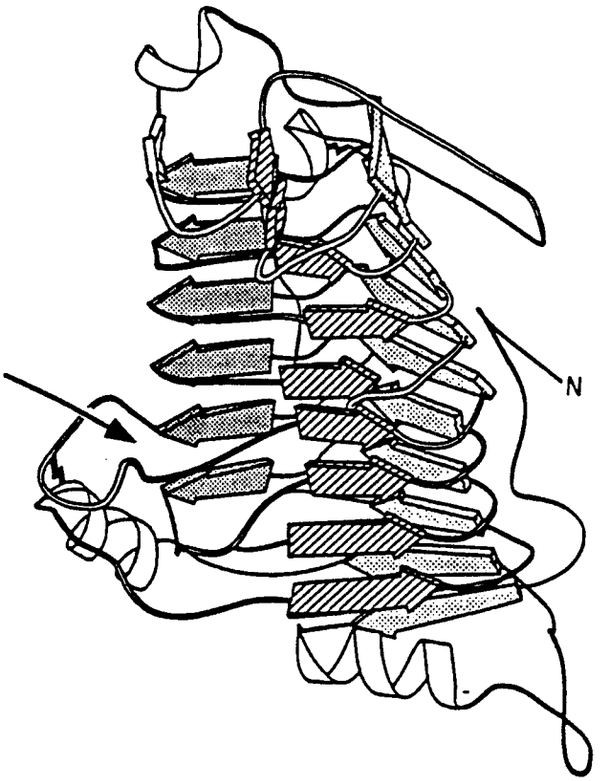


FIGURE 7. Ribbon diagram of the three-dimensional structure of pectate lyase C from *E. chrysanthemi.* The putative catalytic site with a calcium binding site is indicated with an arrow. Reprinted with permission from Yoder et al., 1993a, *Science*, **260**: 1503-07. Copyright 1993 American Association for the Advancement of Science.

(Rees et al., 1982), but also for interactions with the pectate lyase. Various GAG lyases are dependent on calcium, but it is generally not known if this is due to substrate effects or if calcium is also involved in the interaction between GAGs and their lyases. In the case of heparinase I from *F. heparinum,* the presence of homologous calcium binding domain (residue 206 to 213) as part of the heparin binding region suggests a temary interaction between enzyme, substrate and calcium (Sasisekharan et al., 1995a). It is interesting to note that while heparinase II, which has the broadest substrate specificity of the heparinases from *F. heparinum,* is inhibited by calcium, the relatively more specific haparinases I and III have been reported to be activated by calcium. It is possible that calcium is an important determinant of specificity of the heparinases from *F. heparinum.*

E. Mechanism Behind Product Profile

Functional domains of GAG degrading enzymes include binding domains and catalytically active sites. These sites interact with multiple residues of the GAG and lead to cleavage of a glycosidic bond.

1. Kinetic Model for Heparin Degradation

Heparinases specifically recognize certain linkages in heparin (Section III.A.1). This can have an implication for the kinetics of degradation of polymeric heparin; do heparinases degrade cleavable linkages with equal probability or is there a propensity to initiate heparin degradation at certain points?

From the detailed data on product distribution presented in Table 7, a new understanding of the mechanism of cleavage with heparinase I has emerged. Previously, it was believed that heparinase acted endolytically rather than exolytically and that selection among the cleavable linkages was random (Linhardt et al., 1982; Linhardt et al., 1985). When it was realized that tetrasaccharides **4** and **6** consist of cleavable linkages "trapped" in a short oligosaccharide, the ratio of these tetrasaccharides to the final disaccharides could be used to refute this random endolytic model (Cohen and Linhardt, 1990). Also, disaccharide **3** is formed from the very beginning of the reaction, indicating a mechanism that is at least partially exolytic (Rice and Linhardt, 1989). Cohen and Linhardt proposed a rule-based model that was in better agreement with the data (Cohen and Linhardt, 1990). Cleavage takes place either at the nonreducing end of a chain or at the reducing end of one of the main products resistant to heparinase I (disaccharide **1**, tetrasaccharide **5** and hexasaccharide **8**). In the model, the probability of cleavage at each of these sites was proportional to their occurrence. This proposed mechanism suggests a processive sequence of action where the enzyme recognizes 'hot spots' (i.e., the reducing end of non-cleavable linkages) and initiates degradation, cleaving the same chain several times in the direction of the reducing end before it releases and finds a new 'hot spot'. In a separate study, Linhardt found that oligomeric intermediates (hexa- to decamers) each contained exactly one resistant residue (Linhardt et al., 1988a); this observation further supports the model. One inconsistency was found by observing the weight average molecular weight during the course of reaction; this decreased more slowly than predicted by the model when the reaction was from 0 to 20% completed (Cohen and Linhardt, 1990).

2. Product Profiles During Reaction

The evolution of the product profile with time is a valuable source of information on the enzymatic mechanism. Unfortunately, only a few of these kinetic profiles have been published for the GAG lyases and hydrolases (Figure 8) (Michelacci et al., 1987; Rice and Linhardt, 1989). The immediate rise in disaccharide products from the initiation of reaction for both heparinase I and chondroitinase ABC suggest an exolytic action. For heparinase I, however, the result is ambiguous, since the viscosity of the solution drops immediately after onset of reaction, indicating that the chain length is quickly reduced by endo-cleavage (Jandik et al., 1994). The slight S-shape of the disaccharide profile from chondroitinase AC from *F. heparinum* and the significant amount of tetrasaccharides produced indicate a predominantly endolytic activity, supported by the identification of various longer oligosaccharides generated during the reaction (Jandik et al., 1994).

The ambiguity in the interpretation of heparinases exo- or endolytic activity also can be found among the alginate and pectate lyases. Detailed product profile kinetics have been published for these enzymes (Preston and Rice, 1991; Preston et al., 1991; Preston et al., 1992). Because the repeating unit in alginate and pectate is a monosaccharide, the degradation products can have both even and uneven number of monosaccharides. Three types of profiles are reproduced in Figure 9:

In profile type I, many oligosaccharides are formed, some are degraded late in the reaction, and di- and trimer products have S-shaped profiles (Figure 9a). This seems to be an endolytic cleavage pattern.

The type II profile shows an early formation of trimers and short oligomers but only very brief appearance of longer oligomers. The penta- and hexamers are later degraded thereby causing a delayed formation of dimers (Figure 9b). This indicates a mixed type of endolytic/exolytic cleavage, in which intermediate size oligomers are rapidly degraded to trimers.

Finally, the profile type III shows an immediate steep rise in the smallest fragment (dimer), small amounts of shorter oligomers formed, and no larger oligomers detectable (Figure 9c). This seems to be a sort of exolytic mechanism, however, it is surprising that tetra- and pentamers but not trimers are present in the product profile. Thus, it is likely that the mechanism is more complicated than merely exocleavage producing small oligomers. Transglycosylation is one possible mechanism for generating a product profile

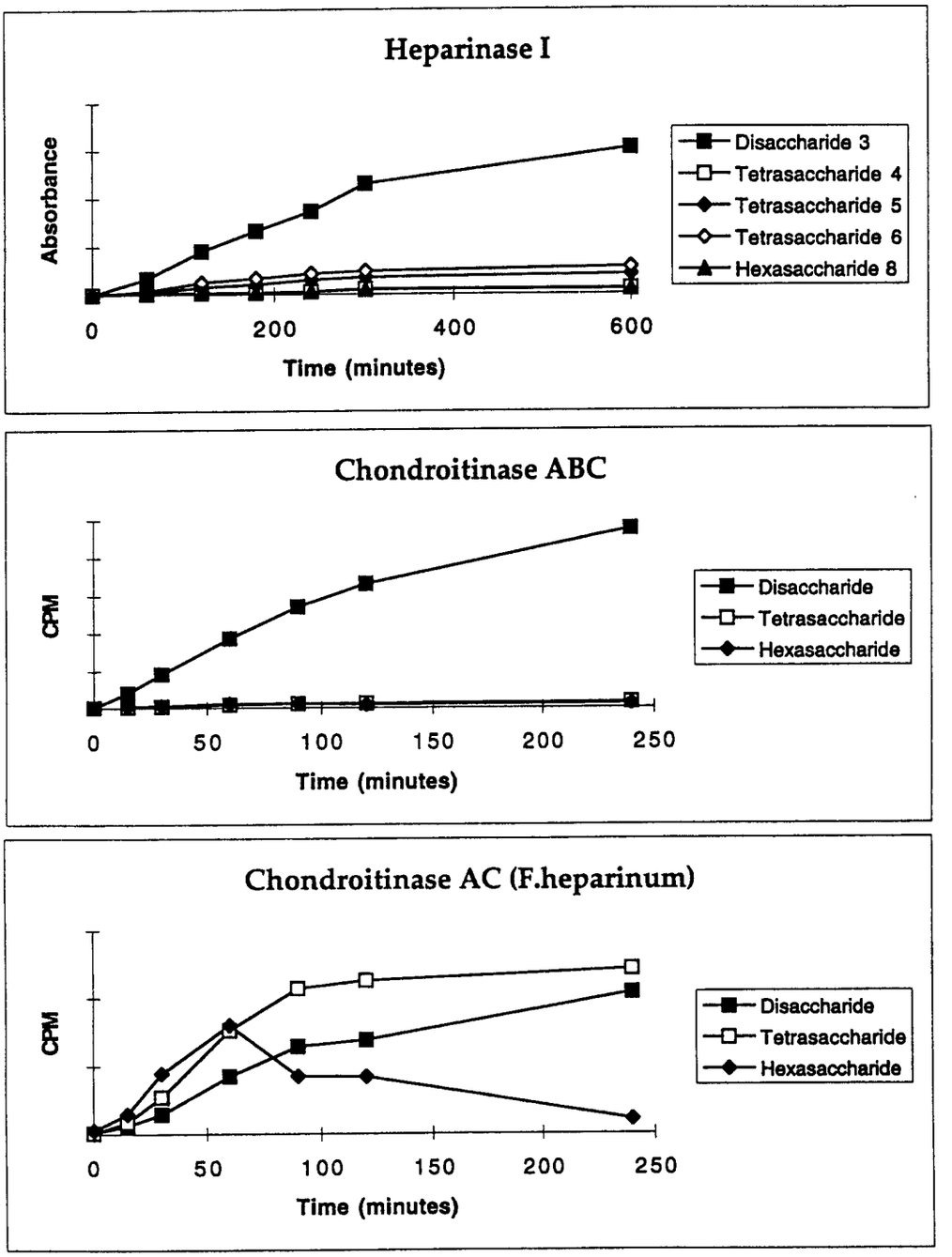


FIGURE 8. Product profiles for heparinase and chondroitinases. Data replotted with permission (Rice and Linhardt, 1989; Michelacci et al., 1987).

in which a certain oligomer appears to be missing. In this respect it is interesting to note that for testicular hyaluronidase, transglycosylation activity could transform hexamers to tetramers and octamers (Highsmith et al., 1975).

Some polysaccharide lyases have a dual mechanism of action. They can both cut endolytically to rapidly reduce the viscosity and produce higher oligomers. They also can cut exolytically in these newly formed oligomers such

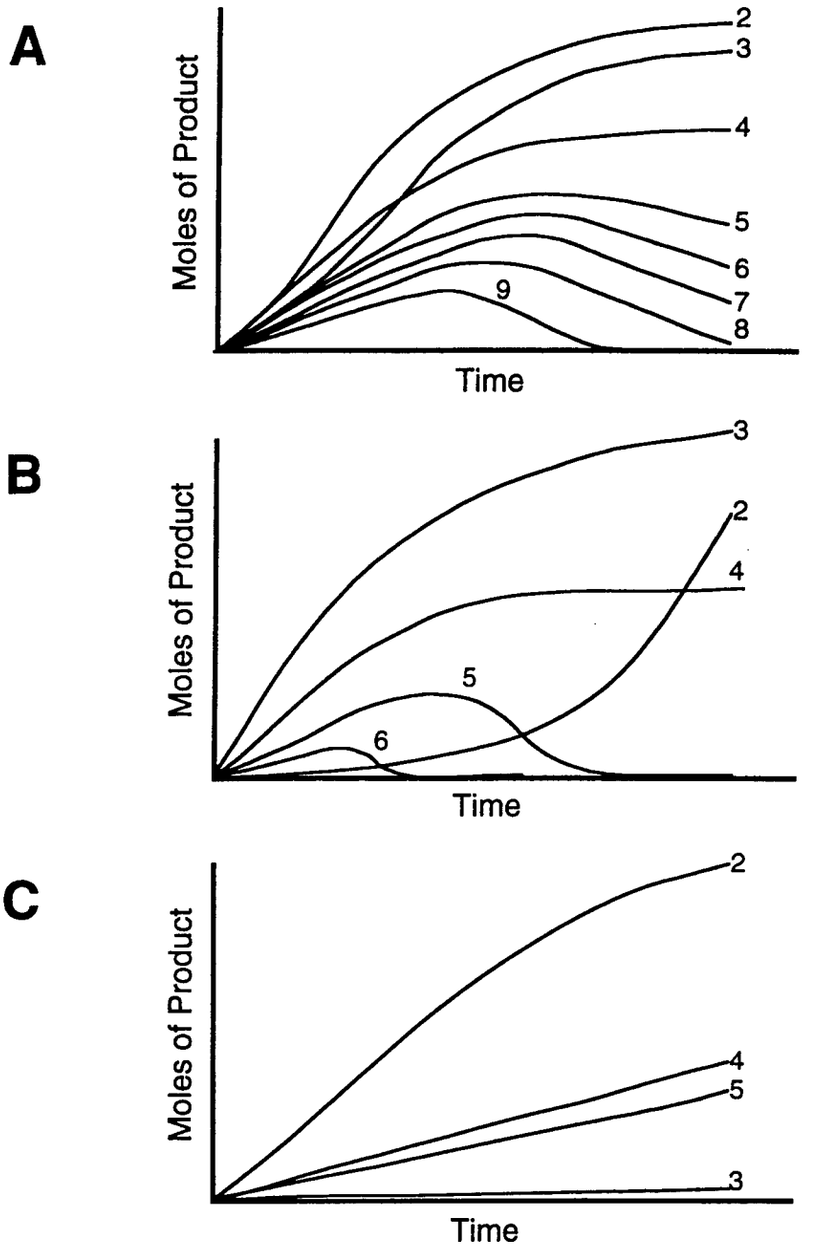


FIGURE 9. Product profiles for pectate and alginate lyases. The numbers indicate the number of monosaccharide residues in the product. Moles of product was determined by UV spectroscopy. A: Product profile type I. B: Product profile type II. C: Product profile type III. See text for discussion of product profiles.

that the small dimeric/trimeric products are generated early in the reaction.

A simple model, presented in Figure 10, can explain why oligomers of certain intermediate sizes are specifically cleaved exolytically. Consider a putative active site with affinity for n residues; each having an intrinsic binding energy, as defined by Jencks (Jencks, 1981), of ΔG_j (free energy/residue). This intrinsic binding energy corresponds to the increase in binding energy

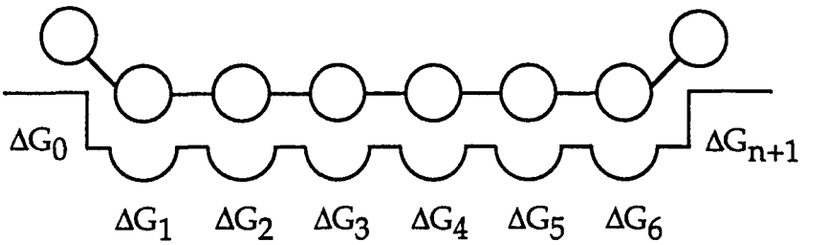


FIGURE 10. Model to explain the mixed endo/exolytic activity which has been observed for GAG lyases as well as for alginate and pectate lyases.

between an oligosaccharide with X-1 residues and one with X residues. For an unmodified GAG such as hyaluronic acid, there is only one value of the binding energy for each subsite. For the modified GAGs such as heparan sulfate, there will be a different binding energy for each combination of subsite and modified disaccharide residue. Assume there is a stereochemical constraint such that the contribution of an extra residue (position '0' or 'n+1'') is positive, counteracting binding. Thus, an oligomer of n residues fits exactly, while longer oligosaccharides will bind less tightly. This mechanism will favor cleavage of an oligomer with n residues instead of the endolytic cleavage of an oligosaccharide with more than n residues. Depending on the position of the scission site, the values of ΔG_i for the individual binding sites and the values of ΔG_0 and ΔG_{n+1}, this model can potentially explain the different product profiles illustrated in Figures 8 and 9. The assignment of binding energies must be done by fitting the model to experimental results.

This approach of assigning binding energies to each site has been applied to starch-degrading enzymes (Allen, 1980); α-amylase from *Bacillus amyloliquefaciens* acts endolytically with preference for the reducing end of amylose. A computer simulation of the product profile showed that a binding pocket, which could accommodate ten saccharide residues with positive ΔG assigned to site number ten was in good agreement with the experimental data (Torgerson et al., 1979; MacGregor and MacGregor, 1985).

Another model to explain the mixed endo/exolytic cleavage was proposed for porcine pancreatic α-amylase: by measuring relative amounts of degradation products from a defined substrate, it was established that each substrate molecule is attacked multiple times by the enzyme during one encounter. Partial enzyme-substrate binding allows one of the oligosaccharide products of the first attack, to "slide" along the binding site and bind in position for a new attack (Robyt and French, 1970; Ward and Moo-Young, 1989). For cleavage of GAGs by lyases, it is possible that the different stereochemistry of the carboxyl group in an unsaturated uronic acid formed by lyase cleavage will enhance the binding affinity to the nonreducing end, thus favoring repeated exolytic cleavage of the same chain.

To fully understand these enzymatic mechanisms, one needs to investigate the molecular interactions between the enzyme and the substrates that lead to favorable binding energies and that bring the catalytically active groups in close contact to the relevant bond. Because some GAGs are heterogeneous polymers, it is important to consider the effect of variations in primary and secondary structure within a chain for binding to and cleavage by enzymes.

IV. SUMMARY

Biopolymer degrading enzymes can be divided in two classes: those that degrade homopolymeric saccharides such as cellulose, starch or chitin, and those that are highly site-specific and degrade heterogeneous, template-synthesized polymers such as proteins and nucleic acids. The GAG depolymerizing enzymes include characteristics of both of these classes. The substrate is heterogeneous, and the enzymes recognize specific residues as is the case for proteases and endonucleases. On the other hand, the substrates are not synthesized from a template; thus, the primary sequence shows higher variability than for a protein or a nucleic acid.

This sequence variability is one of the major problems of GAG and proteoglycan biochemistry. It has hampered many attempts to sequence GAGs or even to determine the average composition. On the contrary, the enzymes that degrade GAG can be studied by all the validated protein techniques of biochemistry and molecular biology. This offers a window of established techniques through which the biochemistry of GAGs and its physiological implications may be investigated.

Four different approaches to elucidate enzyme-GAG interactions have been fruitful recently. Thorough characterization of products formed by enzymatic cleavage, cloning, sequencing and recombinant expression of heparinase I, detailed studies of the structure of the substrate, and finally, crystallographic analysis of the structure of a polysaccharide lyase.

First, extensive biochemical characterization by Linhardt and co-workers of the products of heparinase degradation have established rules for the susceptibility to cleavage of linkages in heparin and heparan sulfate (Linhardt et al., 1992a; Desai et al., 1993a; Desai et al., 1993b).

Second, cloning, sequencing and expression of the heparinase I gene by Sasisekharan allowed determination of a heparin binding site and a catalytically active cysteine (Sasisekharan et al., 1993, 1995a, and 1995b). Continuing along the path of molecular biology is a promising path for proposing, validating and modifying sites of interaction between the enzyme and substrate.

Third, Ragazzi, Casu, and co-workers combined molecular mechanics and two dimensional NMR to study iduronic acid conformers in GAGs (Ragazzi et al., 1986 and 1990). These studies indicated that iduronic acid can attain an equilibrium between the $^{1}C_{4}$, $^{4}C_{1}$ and $^{2}T_{O}$ conformers. This wobbling gives the chain structural flexibility and since the population of these states appears to be dependent on the neighboring residues in the chain, it may also generate specificity in the chain structure. For dermatan sulfate the $^{O}T_{2}$ conformer also may be important (Venkataraman et al., 1994).

Fourth, the recently published crystal structures of pectate lyases (Yoder et al., 1993a and 1993b) may serve as guideline and maybe even a template for solving the three dimensional structure of other lyases. Building a spatial model of the enzyme is a crucial step in simulating and understanding the interactions with the substrate.

These recent advances have increased the knowledge of GAG depolymerization considerably and continuation of this work will enable a more complete understanding of the structural and dynamic features that govern the activity of these enzymes. Thus far, specificities of the enzymes have been defined on the basis of the two saccharides flanking a linkage. It has, however, been shown that the activity of heparinase II and III and keratanase I and II is also dependent on a third saccharide to the reducing and non-reducing side respectively (Yamada et al., 1993, Nakazawa et al., 1989), and that the reaction rate is much lower for cleavage of tetrasaccharides than for cleavage of full length GAG chains (Rice and Linhardt, 1989). Often polysaccharide degrading enzymes like lysozyme and glucoamylases bind four to nine saccharide residues in the catalytic cleft (Kirby, 1987; Belshaw and Williamson, 1993; Sierks et al., 1993). By analogy, it is likely that more saccharides are involved in binding than the two that usually have been used to define specificity of GAG depolymerizing enzymes. This review has summarized recent advances in the study of molecular mechanisms of enzyme-substrate interactions, which are closing the gap between empirical rules of substrate specificity and present knowledge of structure-function relationships for enzymatic degradation of GAGs.

ACKNOWLEDGMENTS

We thank Dr. Ganesh Venkataraman, Prof. Phil Robbins, Dr. Matthew Davis, Ranga Godavarti, Andrew Rhomberg and Chris Dowd for helpful discussions, comments and suggestions. This work was supported by National Institutes of Health (GM 25810) and by National Science Foundation through the Biotechnology Process Engineering Center, MIT.

REFERENCES

Allen, J. D. 1980. Subsite mapping of enzymes: application to polysaccharide depolymerases. *Meth. Enzymol.*, **64**: 248–77.

Ampofo, S. A., Wang, H. M., and Linhardt, R. J. 1991. Disaccharide compositional analysis of heparin and heparan sulfate using capillary zone electrophoresis. *Anal. Biochem.*, **199**: 249–55.

Arnott, S., Guss, J. M., and Hukins, D. W. L. 1974. Conformation of keratan sulphate. *J. Mol. Biol.*, **88**: 175–84.

Ascencio, F., Fransson, L.-Å., and Wadström, T. 1993. Affinity of the gastric pathogen *Helicobacter pylori* for the *N*-sulphated glycosaminoglycan heparan sulphate. *J. Med. Microbiol.*, **38**: 240–44.

Atha, D. H., Lormeau, J.-C., Petitou, M., Rosenberg, R. D., and Choay, J. 1985. Contribuiton of monosaccharide residues in heparin binding to antithrombin III. *Biochemistry*, **24**: 6723–29.

Atkins, E. D. T. and Nieduszynski, I. A. 1976. Heparin: crystalline structures of the sodium and calcium salts. **In**: *Heparin — Chemistry and Clinical Usage*. pp. 21–35. Kakkar, V. V. and Thomas, D. P., Eds., Academic Press, London.

Bailey, J. E. and Ollis, D. F. 1986. *Biochemical Engineering Fundamentals*, McGraw-Hill, New York.

Balduini, C., De Luca, G., and Castellani, A. A. 1989. Biosynthesis of skeletal and corneal keratan sulphate. **In**: *Keratan Sulphate — Chemistry, Biology, Clinical Pathology*. pp. 53–65. Greiling, H. and Scott, J. E., Eds., The Biochemical Society, London.

Bame, K. J. 1993. Release of heparan sulfate glycosaminoglycans from proteoglycans in chinese hamster ovary cells does not require proteolysis of the core protein. *J. Biol. Chem.*, **268**: 19956–64.

Baugh, R. F., Deemar, K. A., and Zimmerman, J. J. 1992. Heparinase in the activated clotting time assay: monitoring heparin-independent alterations in coagulation function. *Anesth. Analg.*, **74**: 201–05.

Bellamy, R. W. and Horikoshi, K. 1992. Heparinase produced by a microorganism belonging to the genus *Bacillus*. *United States Patent*, **5,145,778**.

Bellamy, W. R. 1990. A novel *Bacillus* sp. capable of degrading sulfated glycosaminoglycans. **In**: *Superbugs, Microorganisms in Extreme Environments*. pp. 143–57. Horikoshi, K. and Grant, W. D., Eds., Japan Scientific Societies Press, Tokyo.

Belshaw, N. J. and Williamson, G. 1993. Specificity in the binding domain of glucoamylase 1. *Eur. J. Biochem.*, **211**: 717–24.

Bitter, T. and Muir, H. M. 1962. A modified uronic acid carbazole reaction. *Anal Biochem*, **4**: 330–34.

Böhmer, L. H., Pitout, M. J., Steyn, P. L., and Visser, L. 1990. Purification and characterization of a novel heparinase. *J. Biol. Chem.*, **265**: 13609–17.

Bourin, M.-C. and Lindahl, U. 1993. Glycosaminoglycans and the regulation of blood coagulation. *Biochem. J.*, **289**: 313–30.

Bourin, M.-C., Lundgren-Åkerlund, E., and Lindahl, U. 1990. Isolation and characterization of the glycosaminoglycan component of rabbit thrombomodulin proteoglycan. *J. Biol. Chem.*, **265**: 15424–431.

Cael, J. J., Winter, W. T., and Arnott, S. 1978. Calcium chondroitin 4-sulfate: Molecular conformation and organization of polysaccharide chains in a proteoglycan. *J. Mol. Biol.*, **125**: 21–42.

Cardin, A. D., Demeter, D. A., Weintraub, H. J. R., and Jackson, R. L. 1991. Molecular design and modeling of protein-heparin interactions. *Meth. Enzymol.*, **203**: 556–583.

Cardin, A. D. and Weintraub, H. J. R. 1989. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis*, **9**: 21–32.

Casu, B. 1989. Structure of heparin and heparin fragments. *Ann. N. Y. Acad. Sci.*, **556**: 1–17.

Casu, B., Choay, J., et al. 1986. Controversial glycosaminoglycan conformations. *Nature*, **322**: 215-16.

Casu, B., Petitou, M., Provasoli, M., and Sinaÿ, P. 1988. Conformational flexibility: a new concept for explaining binding and biological properties of iduronic acid-containing glycosaminoglycans. *TIBS*, **13**: 221–25.

Celesia, G. G. 1991. Alzheimer's desease: the proteoglycans hypothesis. *Sem. Thromb. Hem.*, **17 (suppl 2)**: 158–60.

Cerbelaud, E. C., Conway, L. J., Galliher, P. M., Langer, R. S., and Cooney, C. L. 1986. Sulfur regulation of heparinase and sulfatases in *Flavobacterium heparinum*. *Appl. Env. Micr.*, **51**: 640–46.

Cheng, F., Yoshida, K., Heinegård, D., and Fransson, L.-Å. 1992. A new method for sequence analysis of glycosaminoglycans from heavily substituted proteoglycans reveals non-random positioning of 4- and 6-*O*-sulphated *N*-acetylgalactosamine in aggrecan-derived chondroitin sulphate. *Glycobiology*, **2**: 553–61.

Cohen, D. M., and Linhardt, R. J. 1990. Randomness in the heparin polymer: Computer simulations of alternative action patterns of heparin lyase. *Biopolymers*, **30**: 733–41.

Cohen, F. E. 1993. The parallel β helix of pectate lyase C: something to sneeze at. *Science*, **260**: 1444–45.

Coltrini, D., Rusnati, M., et al. 1993. Biochemical bases of the interaction of human basic fibroblast growth factor with the glycosaminoglycans. *Eur. J. Biochem.*, **214**: 51–58.

Comper, W. D. 1981. *Heparin (and related polysaccharides)*, Gordon & Breach, New York.

Conrad, H. E. 1989. Structure of heparan sulfate and dermatan sulfate. *Ann. N.Y. Acad. Sci.*, **556**: 18–28.

Creighton, T. E. 1993. *Proteins: Structures and Molecular Properties* (2. ed.), W.H. Freeman and Co., New York.

D'Amore, P. A. 1990. Modes of FGF release in vivo and in vitro. *Cancer and Metastasis Reviews*, **9**: 227–38.

de Agostini, A. L., Lau, H. K., Leone, C., Youssoufian, H., and Rosenberg, R. D. 1990. Cell mutants defective in synthesizing a heparan sulfate proteoglycan with regions of defined monosaccharide sequence. *Proc. Natl. Acad. Sci., USA*, **87**: 9784–88.

Desai, U. R., Wang, H., and Linhardt, R. J. 1993a. Specificity studies on the heparin lyases from *Flavobacterium heparinum*. *Biochemistry*, **32**: 8140–45.

Desai, U. R., Wang, H., and Linhardt, R. J. 1993b. Substrate specificity of the heparin lyases from *Flavobacterium heparinum*. *Arch. Biochem. Biophys.*, **306**: 461–68.

Dietrich, C. P., Silva, M. E., and Michelacci, Y. M. 1973. Sequential degradation of heparin in *Flavobacterium heparinum*. *J. Biol. Chem.*, **248**: 6408–15.

Engelberg, H. 1991. Heparin, non-heparin glycosaminoglycans, and heparinoids: An overview of their application in atherosclerosis. *Sem. Thromb. Hem.*, **17 (suppl 1)**: 5–8.

Enghild, J. J., Salvesen, G., Hefta, S. A., Thøgersen, I. B., Rutherford, S., and Pizzo, S. V. 1991. Chondroitin 4-sulfate covalently cross-links the chains of the human blood protein pre-α-inhibitor. *J. Biol. Chem.*, **266**: 747–51.

Eriksson, A. E., Cousens, L. S., Weaver, L. H., and Matthews, B. W. 1991. Three-dimensional structure of human basic fibroblast growth factor. *Proc. Natl. Acad. Sci., USA*, **88**: 3441–45.

Ernst, S., Venkataraman, G., Winkler, S., Godavarti, R., Langer, R., Cooney, C. L., and Sasisekharan, R. 1995. Expression, purification, and characterization of heparinase I from *F. heparinum* in *E. coli*. *Submitted to Biochemical J.*,

Evered, D. and Whelan, J. 1986. *Functions of the Proteoglycans*. John Wiley & Sons, Chichester, NY.

Evered, D., and Whelan, J. 1989. *The Biology of Hyaluronan*. John Wiley & Sons, Chichester, NY.

Fedarko, N. S., and Conrad, H. E. 1986. A unique heparan sulfate in the nuclei of hepatocytes: Structural changes with the growth state of the cells. *J. Cell. Biol.*, **102**: 587–99.

Ferro, D. R., Provasoli, A., et al. 1990. Conformer populations of L-iduronic acid residues in glycosaminoglycan sequences. *Carb. Res.*, **195**: 157–67.

Ferro, D. R., Provasoli, A., Ragazzi, M., Meille, S. V., and Colombo, A. 1989. On the solid-state conformation of glycosaminoglycans containing iduronic acid. **In**: *International workshop on recent developments in industrial polysaccharides*. Crescenzi, V., Ed., Gordon and Breach, New York.

Fersht, A. 1985. *Enzyme Structure and Mechanism* (2. ed.), W.H. Freeman and Co., New York.

Folkman, J., and Klagsbrun, M. 1987. Angiogenic Factors. *Science*, **235**: 442–47.

Fransson, L.-Å. 1985. Mammalian Glycosaminoglycans. **In**: *the Polysaccharides*. Aspinall, G. O., Ed., Academic Press, Orlando, FL.

Fransson, L.-Å., and Cöster, L. 1979. Interaction between dermatan sulphate chains. *Biochim. Biophys. Acta*, **582**: 132–44.

Fransson, L.-Å., Havsmark, B., and Sheehan, J. K. 1981. Self-associatin of heparan sulfate. *J. Biol.Chem.*, **256**: 13039–43.

Fransson, L.-Å., Havsmark, B., and Silverberg, I. 1990. A method for the sequence analysis of dermatan sulphate. *Biochem. J.*, **269**: 381–388.

French, A. D., and Brady, J. W. 1990. Computer modeling of carbohydrates. *ACS Symposium Series*, **430**: 1–19.

Gabriel, D. A., and Carr, M. E. 1989. Calcium destabilizes and causes conformational changes in hyaluronic acid. *Am. J. Med. Sci.*, **298**: 8–14.

Gallagher, J. T., Lyon, M., and Steward, W. P. 1986. Structure and function of heparan sulphate proteoglycans. *Biochem. J.*, **236**: 313–25.

Gallagher, J. T., Turnbull, J. E., and Lyon, M. 1992a. Heparan sulphate proteoglycans: molecular organisation of membrane-associated species and an approach to polysaccharide sequence analysis. **In**: *Heparin and related polysaccharides*. Lane, D. A., Björk, I., and Lindahl, U., Eds., Plenum Press, New York.

Gallagher, J. T., Turnbull, J. E., and Lyon, M. 1992b. Patterns of sulphation in heparan sulphate: polymorphism based on a common structural theme. *Int. J. Biochem.*, **24**: 553–60.

Gallagher, J. T., and Walker, A. 1985. Molecular distinctions between heparan sulphate and heparin. *Biochem. J.*, **230**: 665–74.

Gettins, P., and Horne, A. P. 1992. One- and two-dimensional 13 C-n.m.r characterization of two series of oligosaccharides derived from porcine intestinal mucosal heparin by degradation with heparinase. *Carb. Res.*, **223**: 81–98.

Gitay-Goren, H., Soker, S., Vlodavsky, I., and Neufeld, G. 1992. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J. Biol. Chem.*, **267**: 6093–98.

Godder, K., Vlodavsky, I., Eldor, A., Weksler, B. B., Haimonvitz-Friedman, A., and Fuks, Z. 1991. Heparanase activity in cultured endothelial cells. *J. Cell. Physiol.*, **148**: 274–80.

Gospodarowicz, D., and Cheng, J. 1986. Heparin protects basic and acidic FGF from inactivation. *J. Cell. Phys.*, **128**: 475–84.

Greiling, H., and Scott, J. E. 1989. *Keratan Sulphate — Chemistry, Biology, Clinical Pathology*, The Biochemical Society, London.

Greiling, H., Stuhlsatz, W., Eberhard, T., and Eberhard, A. 1975. Studies on the mechanism of hyaluronate lyase action. *Conn. Tissue Res.*, **3**: 135–39.

Grootenhuis, P. D. J. and van Boeckel, C. A. A. 1991. Constructing a molecular model of the interaction between antithrombin III and a potent heparin analogue. *J. Am. Chem. Soc.*, **113**: 2743–47.

Grootenhuis, P. D. J., van Boeckel, C. A. A., and Haasnoot, C. A. G. 1994. Carbohydrates and drug discovery — the role of computer simulation. *TIBTECH*, **12**: 9–14.

Gu, K., Edens, R. E., Jandik, K. A., and Linhardt, R. J. 1993a. Monoclonal antibodies prepared against heparin lyase I and their reactivity toward heparin lyase I, II, and III. *Int. J. Biochem.*, **25**: 331–36.

Gu, K., Liu, J., Pervin, A., and Linhardt, R. J. 1993b. Comparison of the activity of two chondroitin AC lyases on dermatan sulfate. *Carb. Res.*, **244**: 369–77.

Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. 1993. Activating and inhibitory heparin sequences for FGF-2 (Basic FGF). *J. Biol. Chem.*, **268**: 23906–14.

Guo, Y., and Conrad, H. E. 1989. The disaccharide composition of heparins and heparan sulfates. *Anal. Biochem.*, **176**: 96–104.

Guss, J. M., Hukins, D. W. L., et al. 1975. Hyaluronic acid: molecular conformations and interactions in two sodium salts. *J. Mol. Biol.*, **95**: 359–84.

Guthrie, E. P., Shoemaker, N. B., and Salyers, A. A. 1985. Cloning and expression in *Escherichia coli* of a gene coding for a chondroitin lyase from *Bacteroides thetaiotaomicron*. *J. Bactacteriology*, **164**: 510–15.

Haasnoot, C. A. G. 1992. The conformation of six-membered rings described by puckering coordinates derived from endocyclic torsion angles. *J. Am. Chem. Soc.*, **114**: 882–87.

Habuchi, H., Suzuki, S., et al. 1992. Structure of a heparan sulphate oligosaccharide that binds to basic fibroblast growth factor. *Biochem. J.*, **285**: 805–13.

Hamai, A., Morikawa, K., Horie, K., and Tokuyasu, K. 1989. Purification and characterization of hyaluronidase from *Streptococcus dysgalactiae*. *Agric. Biol. Chem.*, **53**: 2163–68.

Hardingham, T. E., and Fosang, A. J. 1992. Proteoglycans: many forms and many functions. *FASEB J*, **6**: 861-70.

Hart, G. W. 1992. Glycosylation. *Curr. Op. Cell. Biol*, **4**: 1017–23.

Hascall, V. C., and Midura, R. J. 1989. Keratan sulphate proteoglycans: chemistry biosynthesis of linkage regions. **In**: *Keratan Sulphate — Chemistry, Biology, Clinical Pathology*. pp. 66–73. Greiling, H. and Scott, J. E., Eds., The Biochemical Society, London.

Hay, E. D. 1991. *Cell Biology of Extracellular Matrix* (2nd ed.), Plenum Press, New York.

Heatley, F., and Scott, J. E. 1988. A water molecule participates in the secondary structure of hyaluronan. *Biochem. J.*, **254**: 489–93.

Highsmith, S., Garvin, J. H., and Chipman, D. M. 1975. Mechanism of action of bovine testicular hyaluronidase. *J Biol Chem*, **250**: 7473–80.

Hinton, J. C. D., Sidebotham, D. R., Gill, G. P., and Solmond, C. 1989. *Mol. Microbiology*, **3**: 1785.

Hirsch, J. 1991. Heparin. *N. Eng. J. Med.*, **324**: 1565–74.

Hiyama, K., and Okada, S. 1975a. Amino Acid composition and physicochemical characterization of chondroitinase from *Arthrobacter aurescens*. *J. Biochem.*, **78**: 1183–90.

Hiyama, K. and Okada, S. 1975b. Crystallization and some properties of chondroitinase from *Arthrobacter aurescens*. *J. Biol. Chem.*, **250**: 1824–28.

Hiyama, K., and Okada, S. 1976. Action of Chondroitinases I. *J Biochem*, **80**: 1201–07.

Hiyama, K, and Okada, S. 1977. Action of Chondroitinases III. *J Biochem*, **82**: 429–36.

Hopwood, J. J. 1989. Enzymes that degrade heparin and heparan sulphate. **In**: *Heparin. — Chemical and biological properties clinical applications*. Lane, D. A. and Lindahl, U., Eds., CRC Press, Boca Raton.

Hounsell, E. F. 1989. Structural and conformational analysis of keratan sulphate oligosaccharides and related carbohydrate structures. **In**: *Keratan Sulphate — Chemistry, Biology, Clinical Pathology*. pp. 12-15. Greiling, H. and Scott, J. E., Eds., The Biochemical Society, London.

Hounsell, E. F., Feeney, J., Scudder, P., Tang, P. W., and Feizi, T. 1986. ^{1}H-NMR studies at 500 MHz of a neutral disaccharide and sulphated di-, tetra-, hexa-, and larger oligosaccharides obtained by endo-β-galactosidase treatment of keratan sulphate. *Eur. J. Biochem.*, **157**: 375–84.

Hunter, G. K., Wong, K. S., and Kim, J. J. 1988. Binding of calcium to glycosaminoglycans: an equilibrium dialysis study. *Arch. Biochem. Biophys.*, **260**: 161–67.

Hwa, V., and Salyers, A. A. 1992. Analysis of two chondroitin sulfate utilization mutants of *Bacteroides thetaiotamicron* that differ in their abilities to compete with the wild type in the gastrointestinal tracts of germfree mice. *Appl. Env. Micr.*, **58**: 869–76.

Ingber, D. E. 1989. Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: Role of extracellular matrix. *J. Cell. Biol.*, **109**: 317–30.

Isaacs, R. D. 1994. *Borrelia burgdorferi* bind to epithelial cell proteoglycans. *J. Clin. Invest.*, **93**: 809–19.

Ishai-Michaeli, R., Eldor, A., and Vlodavsky, I. 1990. Heparanase activity expressed by platelets, neutrophils and lymphoma cells releases active fibroblast growth factor from extracellular matrix. *Cell Regulation*, **1**: 833–42.

Isihara, M., Fedarko, N. S., and Conrad, H. E. 1986. Transport of heparan sulfate into the nuclei of hepatocytes. *J. Biol. Chem.*, **261**: 13575–80.

Isihara, M., Fedarko, N. S., and Conrad, H. E. 1987. Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. *J. Biol. Chem.*, **262**: 4708–16.

Isihara, M., Tyrrell, D. J., Stauber, G. B., Brown, S., Cousens, L. S., and Stack, R. J. 1993. Preparation of affinity-fractionated, heparin-derived oligosaccharides and their effects on selected biological activities mediated by basic fibroblast growth factor. *J. Biol. Chem.*, **268**: 4675–83.

Itoh, K., and Sokol, S. Y. 1994. Heparan sulfate proteoglycans are required for mesoderm formation in *Xenopus* embryos. *Development*, **120**: 2703–11.

Jackson, R. L., Busch, S. J., and Cardin, A. D. 1991. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiological Reviews*, **71**: 481–539.

Jandik, K. A., Gu, K., and Linhardt, R. J. 1994. Action patterns of polysaccharide lyases on glycosaminoglycans. *Glycobiology*, **4**: 289–96.

Jaye, M., Schlessinger, J., and Dionne, C. A. 1992. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochim. Biophys. Acta*, **1135**: 185–99.

Jencks, W. P. 1981. On the attribution and additivity of binding energies. *Proc. Natl. Acad. Sci., USA*, **78**: 4046–50.

Joubert, J. J., and Pitout, M. J. 1985. A constitutive heparinase in a *Flavobacterium* sp. *Experientia*, **41**: 1541.

Juhasz, P., and Biemann, K. 1994. Mass spectrometric molecular weight determination of highly acidic compounds

of biological significance via their complexes with basic polypeptides. *Proc. Natl. Acad, Sci., USA*, **91**: 4333–37.

Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeehan, W. L. 1993. An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science*, **259**: 1918–21.

Kemeney, D. M., Dalton, N., Lawrence, A. J., Pearce, F. L., and Vernon, C. A. 1984. The purification and characterization of hyaluronidase from the venom of the honey bee, *Apis mellifera*. *Eur. J. Biochem.*, **139**: 217–23.

Kiefer, M. C., Ishihara, M., Swiedler, S. J., Crawford, K., Stephans, J. C., and Barr, P. J. 1991. The molecular biology of heparan sulfate fibroblast growth factor receptors. *Ann. N.Y. Acad. Sci.*, **556**:

Kirby, A. J. 1987. Mechanism and stereoelectronic effects in the lysozyme reaction. *CRC Crit. Rev. Biochem.*, **22**: 283–312.

Kitamikado, M., and Lee, Y.-Z. 1975. Chondroitinase-producing bacteria in natural habitats. *Appl. Microbiol.*, **29**: 414–21.

Kjellèn, L., and Lindahl, U. 1991. Proteoglycans: structures and interactions. *Ann. Rev. Biochem.*, **60**: 443–75.

Klagsbrun, M., and Baird, A. 1991. A dual receptor system is required for basic fibroblast growth factor activity. *Cell*, **67**: 229–31.

Kojima, T., Leone, C. W., Marchildon, G. A., Marcum, J. A., and Rosenberg, R. D. 1992. Isolation and characterization of heparan sulfate proteoglycans produced by cloned rat microvascular endothelial cells. *J. Biol. Chem.*, **267**: 4859–69.

Kresse, H. 1989. Degradation of keratan sulphate proteoglycans. **In**: *Keratan Sulphate — Chemistry, Biology, Clinical Pathology*. pp. 111–121. Greiling, H. and Scott, J. E., Eds., The Biochemical Society, London.

Kresse, H., and Glossl, J. 1987. Glycosaminoglycan degradation. *Adv. Enzym.*, **60**: 217–312.

Kvam, B. J., Atzori, M., Toffanin, R., Paoletti, S., and Biviano, F. 1992. 1H and 13C NMR studies of solutions of hyaluronic acid esters and salts in methyl sulfoxide: comparison of hydrogen-bond patterns and conformational behaviour. *Carb. Res.*, **230**: 1–13.

Lane, D. A., and Lindahl, U., Eds. 1989. *Heparin — chemical and biological properties clinical applications*. CRC Press, Boca Raton.

Langer, R., Linhardt, R. J., et al. 1982. An enzymatic system for removing heparin in extracorporeal therapy. *Science*, **217**: 261–63.

Laskov, R., Michaeli, R. I., Sharir, H., Yefenof, E., and Vlodavsky, I. 1991. Production of heparanase by normal and neoplastic murine B-lymphocytes. *Int. J. Cancer*, **47**: 92–98.

Lee, M. K., and Lander, A. D. 1991. Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: development of a sensitive electrophoretic approach. *Proc. Natl. Acad. Sci., USA*, **88**: 2768–72.

Lindahl, U. 1989. Biosynthesis of heparin and related polysaccharides. **In**: *Heparin — Chemical and biological properties clinical applications*. pp. 159-91. Lane, D. A. and Lindahl, U., Eds., CRC Press, Boca Raton.

Lindahl, U., Kusche, M., Lidholt, K., and Oscarrson, L.-G. 1989. Biosynthesis of heparin and heparan sulfate. *Ann. N. Y. Acad. Sci.*, **556**: 36–50.

Lindahl, U., Lidholt, K., Spillmann, D., and Kjellén, L. 1994. More to "heparin" than anticoagulation. *Thrombosis Research*, **75**: 1–32.

Lindahl, U., Thinberg, L., Bäckstrom, G., Riesenfeld, J., Nordling, K., and Björk, I. 1984. Extension and structural variability of the antithrombin-binding sequence in heparin. *J. Biol. Chem.*, **259**: 12368–76.

Lindblom, A., Bengtsson-Olivecrona, G., and Fransson, L.-Å. 1991. Domain structure of endothelial heparan sulphate. *Biochem. J.*, **279**: 821–29.

Linhardt, R. J. 1994. Analysis of glycosaminoglycans with polysaccharide lyases. **In**: *Current Protocols in Molecular Biology, Chapter 17 suppl.* Varki, A., Ed.

Linhardt, R. J., Al-hakim, A., et al. 1991. Structural features of dermatan sulfates and their relationship to anticoagulant and antithrombotic activities. *Biochem. Pharm.*, **42**: 1609–19.

Linhardt, R. J., Ampofo, S. A., Fareed, J., Hoppensteadt, D., Mulliken, J. B., and Folkman, J. 1992. Isolation and characterization of human heparin. *Biochemistry*, **31**: 12441–45.

Linhardt, R. J., Fitzgerald, G. L., Cooney, C. L., and Langer, R. 1982. Mode of action of heparin lyase on heparin. *Biochim. Biophys. Acta.*, **702**: 197–203.

Linhardt, R. J., Galliher, P. M., and Cooney, C. L. 1986. Polysaccharide lyases. *Appl. Biochem. Biotech.*, **12**: 135–75.

Linhardt, R. J., Merchant, Z. M., et al. 1985. Evidence of random structural features in the heparin polymer. *Biochemistry*, **24**: 7805–10.

Linhardt, R. J., Rice, K. G., Kim, Y. S., Engelken, J. D., and Weiler, J. M. 1988a. Homogeneous, structurally defined heparin-oligosaccharides with low anticoagulant activity inhibit the generation of the amplification pathway C3 convertase in vitro. *J. Biol. Chem.*, **263**: 13090–96.

Linhardt, R. J., Rice, K. G., Kim, Y. S., Lohse, D. L., Wang, H. M., and Loganathan, D. 1988b. Mapping and quantification of the major oligosaccharide components of heparin. *Biochem. J.*, **254**: 781–87.

Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D., and Gallagher, J. T. 1990. Examination of the substrate specificity of heparin and heparan sulfate lyases. *Biochemistry*, **29**: 2611–17.

Linhardt, R. J., Wang, H.-M., Loganathan, D., and Bae, J.-H. 1992a. Search for the heparin antithrombin III-binding precursor. *J. Biol. Chem.*, **267**: 2380–87.

Linhardt, R. J., Wang, H. M., and Ampofo, S. A. 1992b. New methodologies in heparin structure analysis and the generation of LMW heparins. **In**: *Heparin and related polysaccharides*. Lane, D. A., Björk, I., and Lindahl, U., Eds., Plenum Press, New York.

Linker, A., and Hovingh, P. 1972. Heparinase and heparitinase from Flavobacteria. *Meth. Enzymol.*, **28**: 902–11.

Linn, S., Chan, T., Lipeski, L., and Salyers, A. A. 1983. Isolation and characterization of two chondroitin lyases from *Bacteroides thetaiotamicron*. *J Bacteriology*, **156**: 859–66.

Loganathan, D., Wang, H. M., Mallis, L. M., and Linhardt, R. J. 1990. Structural variation in the antithrombin III binding site region and its occurence in heparin from different sources. *Biochemistry*, **29**: 4362–68.

Lohse, D. L., and Linhardt, R. J. 1992. Purification and characterization of heparin lyases from *Flavobacterium heparinum*. *J. Biol. Chem.*, **267**: 23347–55.

Lortat-Jacob, H., and Grimaud, J.-A. 1992. Binding of interferon-gammma to heparan sulfate is restricted to the heparin-like domains and involves carboxylic — but not *N*-sulfated — groups. *Biochim. Biophys. Acta*, **1117**: 126–30.

Lyon, M., Deakin, J. A., Mizuno, K., Nakamura, T., and Gallagher, J. T. 1994. Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. *J. Biol. Chem.*, **269**: 11216–23.

Maccarana, M., Casu, B., and Lindahl, U. 1993. Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J. Biol. Chem.*, **268**: 23898–905.

MacGregor, E. A., and MacGregor, A. W. 1985. A model for the action of cereal alpha amylases on amylose. *Carb. Res.*, **142**: 223–36.

Maimone, M. M., and Tollefsen, D. M. 1990. Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. *J. Biol. Chem.*, **265**: 18263–71.

Marcum, J. A., and Rosenberg, R. D. 1989a. The biochemistry, cell biology, and patophysiology of anticoagulantly active heparin-like molecules of the vessel wall. **In**: *Heparin — chemical and biological properties clinical applications*. Lane, D. A. and Lindahl, U., Eds., CRC Press, Boca Raton.

Marcum, J. A., and Rosenberg, R. D. 1989b. Role of endothelial cell surface heparin-like polysaccharides. *Ann. N.Y. Acad. Sci.*, **556**: 81–94.

Mascarelli, F., G, F., and Courtois, Y. 1993. aFGF binding to low and high affinity receptors induces both aFGF and aFGF receptors dimerization. *Growth Factors*, **8**: 211–33.

Matheson, N. K., and McCleary, B. V. 1985. Enzymes metabolizing polysaccharides and their application to the analysis of structure and function of glycans. **In**: *the polysaccharides*. Aspinall, G. O., Ed., Academic Press, Orlando.

Matzner, Y., Vlodavsky, I., Bar-Ner, M., Ishai-Michaeli, R., and Tauber, A. I. 1992. Subcellular localization of heparanase in human neutrophils. *J. Leukoc. Biol.*, **51**: 519–24.

McLean, M. W., Long, W. F., and Williamson, F. B. 1985. *Proc. 8th Int. Symp. Glycoconjugates*, pp. 73–74.

Michelacci, Y. M., and Dietrich, C. P. 1975. A comparative study between a chondroitinase B and a chondroitinase AC from *Flavobacterium heparinum*. *Biochem. J.*, **151**: 121–29.

Michelacci, Y. M., and Dietrich, C. P. 1976. Chondroitinase C from *Flavobacterium heparinum*. *J. Biol. Chem.*, **254**: 1154–58.

Michelacci, Y. M., Horton, D. S. P. Q., and Poblacion, C. A. 1987. Isolation and characterization of an induced chondroitinase ABC from *Flavobacterium heparinum*. *Biochim. Biophys. Acta*, **923**: 291–301.

Millane, R. P., Mitra, K., and Arnott, S. 1983. Chondroitin 4-sulfate: comparison of the structures of the potassium and sodium salts. *J. Mol. Biol.*, **169**: 903–20.

Mitra, A. K., Arnott, S., Atkins, E. D. T., and Isaac, D. H. 1983. Dermatan sulfate: molecular conformations and interactions in the condensed state. *J. Mol. Biol.*, **169**: 873–901.

Moffat, C. F., McLean, M. W., Long, W. F., and Williamson, F. B. 1991a. Heparinase II from *Flavobacterium heparinum* — action on chemically modified heparins. *Eur. J. Biochem.*, **197**: 449–59.

Moffat, C. F., McLean, M. W., Long, W. F., and Williamson, F. B. 1991b. Heparinase II from *Flavobacterium heparinum*: HPLC analysis of the saccharides generated from chemically modified heparins. *Eur. J. Biochem.*, **202**: 531–41.

Morris, E. R., Rees, D. A., and Welsh, E. J. 1980. Conformation and dynamic interactions in hyaluronate solutions. *J. Mol. Biol.*, **138**: 383–400.

Mourey, L., Samama, J.-P., Delarue, M, Petitou, M., Choay, J., and Moras, D. 1993. Crystal structure of cleaved bovine antithrombin III at 3.2 Å resolution. *J. Mol. Biol.*, **232**: 223–41.

Mulloy, B., Forster, M. J., Jones, C., and Davies, D. B. 1993. N.m.r. and molecular-modeling studies of the solution conformation of heparin. *Biochem. J.*, **293**: 849–58.

Murata, K., and Yokoyama, Y. 1987. Characterization of the products generated from oversulphated dermatan sulphate isomers with chondroitinase-B high-performance liquid chromatography. *J. Chrom.*, **423**: 51–61.

Nader, H. B., and Dietrich, C. P. 1989. Natural occurence and possible biological role of heparin. **In**: *Heparin — Chemical and biological properties clinical applications*. pp. 81–96. Lane, D. A. and Lindahl, U., Eds., CRC Press, Boca Raton.

Nader, H. B., Dietrich, C. P., Buonassi, V., and Colburn, P. 1987. Heparin sequence in the heparan sulfate chains of an endothelial cell proteoglycan. *Proc. Natl. Acad. Sci., USA*, **84**: 3565–69.

Nader, H. B., Porcionatto, A., et al. 1990. Purification and substrate specificity of heparitinase I and heparitinase II from *Flavobacterium heparinum*. *J. Biol. Chem.*, **265**: 16807–13.

Nakada, H. I., and Wolfe, J. B. 1961. Studies on the enzyme chondroitinase: product structure and ion effects. *Arch. Biochem. Biophys.*, **94**: 244–51.

Nakagawa, H., Yamada, T., et al. 1980. Isolation and characterization of an endo-β-galactosidase from a new strain of *Escherichia freundii*. *J. Biol. Chem.*, **255**: 5955–59.

Nakajima, M., Irimura, T., Ferrante, N. D., and Nicolson, G. L. 1984. Metastatic melanoma cell heparanase. *J. Biol. Chem.*, **259**: 2283–90.

Nakajima, M., Irimura, T., and Nicolson, G. L. 1986. Tumor meastasis-associated heparanase activity in human melanoma cells. *Cancer Lett.*, **31**: 277–83.

Nakajima, M., Irimura, T., and Nicolson, G. L. 1988. Heparanases and tumor metastasis. *J Cell. Biochem.*, **36**: 157–67.

Nakamura, T., Shibata, Y., and Fujimura, S. 1988. Purification and properties of *Bacteroides heparinolyticus* heparinase. *J. Clin. Microbiol.*, **26**: 1070–71.

Nakazawa, K., Ito, M., Yamagata, T., and Suzuki, S. 1989. Substrate specificity of keratan sulphate-degrading enzymes from microorganisms. **In**: *Keratan Sulphate — Chemistry, Biology, Clinical Pathology*. pp. 99–110. Greiling, H. and Scott, J. E., Eds., The Biochemical Society, London.

Nelson, R., Ceccconi, O., Roberts, W., Aruffo, A., Linhardt, R., and Bevilacqua, M. 1993. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood*, **82**: 3253–58.

Nieduszynski, I. A. 1985. Connective tissue polysaccharides. **In**: *Polysaccharides — Topics in structure and morphology*. Atkins, E. D. T., Ed., VCH, Weinheim, Germany.

Nieduszynski, I. A., Huckerby, T. N., Dickenson, J. M., Orown, G. M., Gui-Hua, T., and Bayliss, M. T. 1990. Structural aspects of skeletal keratan sulphates. *Biochem. Soc. Trans.*, **18**: 792–93.

Norgard-Sumnicht, K., and Varki, A. 1995. Endothelial heparan sulfate proteoglycans that bind to L-selectin have glucosamine residues with unsubstituted amino groups. *J. Biol. Chem.*, **270**: 12012–24.

Norgard-Sumnicht, K. E., Varki, N. M., and Varki, A. 1993. Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells. *Science*, **261**: 480–83.

Oeben, Keller, R., Stuhlsatz, H. W., and Greiling, H. 1987. Constant and variable domains of different disaccharide structure in corneal keratan sulphate chains. *Biochem. J.*, **248**: 85–93.

Ogamo, A., Nagai, A., and Nagasawa, K. 1985. Binding of heparin fractions and other polysulfated polysaccharides to plasma fibronectin: effects of molecular size and degree of sulfation of polysaccharides. *Biochim. Biophys. Acta*, **841**: 30–41.

Oliviero, U., Sorrentino, G. P., et al. 1991. Effects of the treatment with matrix on elderly people with chronic articular degeneration. *Drugs. Exp. Clin. Res.*, **17**: 45–51.

Oosta, G. M., Favreau, L. V., Beeler, D. L., and Rosenberg, R. D. 1982. Purification and properties of human platelet heparitinase. *J Biol Chem*, **257**: 11249–55.

Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svahn, C.-M., and Waksman, G. 1995. FGF binding and FGF receptor activation by synthetic heparin-derived di- and tri-saccharides. *Science*, **268**: 432–36.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., and Leder, P. 1992. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell. Biol.*, **12**: 240–47.

Pantoliano, M., Horlick, R. A., et al. 1994. Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. *Biochemistry*, **33**: 10229–48.

Parthasarathy, N., Goldberg, I. J., Sivaram, P., Mulloy, B., Flory, D. M., and Wagner, W. D. 1994. Oligosaccharide sequences of endothelial cell surface heparan sulfate proteoglycan with affinity for lipoprotein lipase. *J. Biol. Chem.*, **269**: 22391–96.

Pejler, G., Bäckström, G., et al. 1987a. Structure and affinity for antithrombin of heparan sulfate chains derived from basement membrane proteoglycans. *J. Biol. Chem.*, **262**: 5036–43.

Pejler, G., Danielsson, Å., Björk, I., Lindahl, U., Nader, H. B., and Dietrich, C. P. 1987b. Structure and antithrombin-binding properties of heparin isolated from the clams *Anomalocardia brasiliana* and *Tivela mactroides*. *J. Biol. Chem.*, **262**: 11413–21.

Pickersgill, R., Jenkins, J., Harris, G., Nasser, W., and Robert-Baudouy, J. 1994. The structure of *Bacillus subtilis* pectate lyase in complex with calcium. *Structural Biology*, **1**: 717–23.

Poole, A. R. 1986. Proteoglycans in health and disease: structure and functions. *Biochem. J.*, **236**: 1–14.

Prehm, P. 1983. Synthesis of hyaluronate in differentiated teratocarcinoma cells. *Biochem. J.*, **211**: 191–98.

Prehm, P. 1984. Hyaluronate is synthesized at plasma membranes. *Biochem. J.*, **220**: 597–600.

Preston, J. F., and Rice, J. D. 1991. Kinetic analysis of pectate lyases by high-performance liquid chromatography. *Carb. Res.*, **215**: 137–45.

Preston, J. F., Rice, J. D., Chow, M. C., and Brown, B. J. 1991. Kinetic comparisons of trimer-generating pectate and alginate lyases by reversed-phase ion-pair liquid chromatography. *Carb. Res.*, **215**: 147–57.

Preston, J. F., Rice, J. D., Ingram, L. O., and Keen, N. T. 1992. Differential depolymerization mechanisms of pectate lyases secreted by *Erwinia chrysanthemi* EC16. *J. Bact.*, **174**: 2039–42.

Prestrelski, S. J., Fox, G. M., and Arakawa, T. 1992. Binding of heparin to basic fibroblast growth factor induces a conformational change. *Arch. Biochem. Biophys.*, **293**: 314–19.

Ragazzi, M., Ferro, D. R., Perly, B., Sinaÿ, P., Petitou, M., and Choay, J. 1990. Conformation of the pentasaccharide corresponding to the binding site of heparin for antithrombin III. *Carb. Res.*, **195**: 169–85.

Ragazzi, M., Ferro, D. R., and Provasoli, A. 1986. A force-field study of the conformational characteristics of the iduronate ring. *J. Comp. Chem.*, **7**: 105–112.

Rapraeger, A. C. 1993. The coordinated regulation of heparan sulfate, syndecans and cell behavior. *Curr. Op. Cell Biol.*, **5**: 844–53.

Rapraeger, A. C., Krufka, A., and Olwin, B. B. 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science*, **252**: 1705–08.

Rautela, G. S., and Abramson, C. 1973. Crystallization and partial characterization of *Staphylococcus aureus*

hyaluronate lyase. *Arch. Biochem. Biophys.*, **158**: 687–94.

Rees, D. A., Morris, E. R., Stoddart, J. F., and Stevens, E. S. 1985. Controversial glycosaminoglycan conformations. *Nature*, **317**: 480.

Rees, D. A., Morris, E. R., Thom, D., and Madden, J. K. 1982. Shapes and interactions of carbohydrate chains. **In**: *the Polysaccharides*. pp. Aspinall, G. O., Ed., Academic Press, New York.

Rice, K. G., and Linhardt, R. J. 1989. Study of structurally defined oligosaccharide substrates of heparin and heparan monosulfate lyases. *Carb. Res.*, **190**: 219–33.

Robyt, J. F., and French, D. 1970. Multiple attack and polarity of action of procine pancreatic α-amylase. *Arch. Biochem. Biophys.*, **138**: 662–70.

Roden, L., Cambell, P., et al. 1989. Enzymic pathways of hyaluronan catabolism. **In**: *the Biology of Hyaluronan*. pp. 60–86. Ciba Foundation, John Wiley & Sons, Chichester, NY.

Rosenberg, R. D. 1989. Platelet Heparitinase. *Meth. Enzym.*, **169**: 342–351.

Rosengart, T. K., Johnson, W. V., Friesel, T., Clark, T., and Maciag, T. 1988. Heparin protects heparin-binding growth factor-I from proteolytic inactivation in vitro. *Biochem. Biophys. Res. Comm.*, **152**: 432–40.

Saksela, O., Moscatelli, D., Sommer, A., and Rifkin, D. B. 1988. Endorthelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. *J. Cell. Biol.*, **107**: 743–51.

Salyers, A. A., Vercellotti, J. R., West, S. E. H., and Wilkins, T. D. 1977. Fermentation of mucin and plant polysaccharides by strains of bacteroides from the human colon. *Appl. Environ. Microbiol.*, **33**: 319–22.

Sanderson, P. N., Huckerby, T. N., and Nieduszynski, I. A. 1989. Chondroitinase ABC digestion of dermatan sulphate. *Biochem. J.*, **257**: 347–54.

Sandström, J., Carlsson, L., Marklund, S. L., and Edlund, T. 1992. The heparin-binding domain of extracellular superoxide dismutase C and formation of variants with reduced heparin affinity. *J. Biol. Chem.*, **267**: 18205–09.

Sasisekharan, R. 1991. *Cloning and biochemical characterization of heparinase from Flavobacterium heparinum*. Ph.D thesis, Harvard University.

Sasisekharan, R., Bulmer, M., Moremen, K. W., Cooney, C. L., and Langer, R. 1993. Cloning and expression of heparinase I gene from *Flavobacterium heparinum*. *Proc. Natl. Acad. Sci., USA*, **90**: 3660–64.

Sasisekharan, R., Godavarti, R., Ernst, S., Venkataraman, G., Cooney, C. L., and Langer, R. 1995a. Characterization and mapping of heparinase I binding to heparin: identification of a heparin binding sequence. *Submitted to J. Biol. Chem.*

Sasisekharan, R., Leckband, D., Godavarti, R., Venkataraman, G., Cooney, C. L., and Langer, R. 1995b. Heparinase I from *Flavobacterium heparinum*. The role of the cysteine residue in catalysis as probed by chemical modification and site-directed mutagenesis. *Biochemistry*, **34**: In press.

Sasisekharan, R., Moses, M. A., Nugent, M. A., Cooney, C. L., and Langer, R. 1994. Heparinase inhibits neovascularization. *Proc. Natl. Acad. Sci., USA*, **91**: 1524–28.

Sato, N., Murata, K., and Kimura, A. 1986. Subunit structure of chondroitinase ABC from *Proteus vulgaris*. *Agric. Biol. Chem.*, **50**: 1057–59.

Savion, N., Disatnik, M. H., and Nevo, Z. 1987. Murine macrophage heparanase: inhibition and comparison with metastatic tumor cells. *J. Cell. Physiol.*, **130**: 77–84.

Sawaguchi, S., Yue, B., Yeh, P., and Tso, M. O. M. 1992. Effects of intracameral injection of chondroitinase ABC in vivo. *Arch. Ophthalmol.*, **110**: 110–17.

Schreuder, H. A., de Boer, B., et al. 1994. The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions. *Nature Structural Biology*, **1**: 48–54.

Scott, J. E. 1992. Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues. *FASEB J*, **6**: 2639–45.

Scott, J. E., Chen, Y., and Brass, A. 1992. Secondary and tertiary structures involving chondroitin and chondroitin sulphates in solution investigated by rotary shadowing/electron microscopy and computer simulation. *Eur. J. Biochem.*, **209**: 675–80.

Scott, J. E., Cummings, C., Brass, A., and Chen, Y. 1991. Secondary and tertiary structures of hyaluronan in aqueous solution investigated by rotary shadowing/electron microscopy and computer simulation. *Biochem. J.*, **274**: 699–705.

Scott, J. E., Heatley, F., Jones, M. N., Wilkinson, A., and Olavesen, A. H. 1983. Secondary structure of chondroitin sulphate in dimethyl sulphoxide. *Eur. J. Biochem.*, **130**: 491–95.

Scudder, P., Tang, P. W., Hounsell, E. F., Lawson, A. M., Mehmet, H., and Feizi, T. 1986. Isolation and characterization of sulphated oligosaccharides released from bovine corneal keratan sulphate by the action of endo-β-galactosidase. *Eur. J. Biochem.*, **157**: 365–73.

Sheehan, J. K., Gardner, K. H., and Atkins, E. D. T. 1977. Hyaluronic acid: a double-helical structure in the presence of potassium at low pH and found also with the cations ammonium, rubidium and caesium. *J. Mol. Biol.*, **117**: 113–35.

Shieh, M.-T., WuDunn, D., Montgomeryu, R. I., Esko, J. D., and Spear, P. G. 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biology*, **116**: 1273–81.

Sierks, M. R., Ford, C., Reilly, P. J., and Svensson, B. 1993. Functional roles and subsite locations of Leu177, Trp 178 and Asn182 of *Aspergillus awamori* glucoamylase determined by site-directed mutagenesis. *Protein Eng*, **6**: 75–79.

Silva, M. E., Dietrich, C. P., and Nader, H. B. 1976. On the structure of heparitin sulfates. *Biochim. Biophys. Acta.*, **437**: 129–41.

Silverberg, I., Havsmark, B., and Fransson, L.-Å. 1985. The substrate specificity of heparin sulphate lyase and heparin from *Flavobacterium heparinum*. *Carb. Res.*, **137**: 227–38.

Sjøgren, P., Pedersen, T., and Steinmetz, H. 1987. Mucopolysaccharidoses and anaesthetic risks. *Acta. Anaesthesiol. Scand.*, **31**: 214–18.

Sommer, A., and Rifkin, D. B. 1989. Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J. Cell. Phys.*, **138**: 215–20.

Sporn, M. B., and Roberts, A. B. 1990. *Peptide Growth Factors and Their Receptors*, Springer-Verlag, Berlin.

Springer, B. A., Pantoliano, M. W., et al. 1994. Identification and concerted function of two receptor binding sufaces on basic fibroblast growth factor required for mitogenesis. *J. Biol. Chem.*, **269**: 26879–84.

Steyn, P. L., Pot, B., Segers, P., Kersters, K., and Joubert, J. J. 1992. Some novel aerobic heparin-degrading bacterial isolates. *System. Appl. Microbiol.*, **15**: 137–43.

Stoddart, J. F. 1971. *Stereochemistry of carbohydrates*, John Wiley and Sons, New York.

Stuhlsatz, H. W., Hirtzel, F., Keller, R., Cosma, S., and Greiling, H. 1981. Studies on the polydispersity and heterogeneity of proteokeratan sulfate from calf and porcine cornea. *Hoppe-Seyler's Z. Physiol Chem*, **362**: 841–52.

Stuhlsatz, H. W., Keller, R., and al., e. 1989. Strucuture of keratan sulphate proteoglycans: core proteins, linkage regions, carbohydrate chains. **In**: *Keratan Sulphate — Chemistry, Biology, Clinical Pathology*. pp. 1–11. Greiling, H. and Scott, J. E., Eds., The Biochemical Society, London.

Takegawa, K., Iwahara, K., and Iwahara, S. 1991. Purification and properties of chondroitinase produced by a bacterium isolated from soil. *J. Ferm. Bioeng.*, **72**: 128–31.

Tam, Y.-C., and Chan, E. C. S. 1985. Purification and characterization of hyaluronidase from oral *Peptostreptococcus* species. *Infect. Imm.*, **47**: 508–13.

Taylor, R. L., Shively, J. E., Conrad, H. E., and Cifonelli, J. A. 1973. Uronic acid composition of heparins and heparan sulfates. *Biochemistry*, **12**: 3633–37.

Tejidor, L., Oman, D., et al. 1993. Use of DADE hepzyme to eliminate heparin interference in coagulation testing. *Thromb. Hemostasis*, **69**: 866.

Thompson, L. D., Pantoliano, M. W., and Springer, B. A. 1994. Energetic characterization of the basic fibroblast growth factor-heparin interaction: Identification of the heparin binding domain. *Biochemistry*, **33**: 3831–40.

Thornton, D. J., Morris, H. G., et al. 1989. Structural and immunological studies of keratan sulphates from mature bovine articular cartilage. *Biochem. J.*, **260**: 277–82.

Thunberg, L., Backstrom, G., Wasteson, Å., Robinson, H. C., Ogren, S., and Lindahl, U. 1982. Enzymatic depolymerization of heparin-related polysaccharides. *J. Biol. Chem.*, **257**: 10278–82.

Thurston, C. F., Hardingham, T. E., and Muir, H. 1975. The kinetics of degradation of chondroitin sulphates and hyaluronic acid by chondroitinase from *Proteus vulgaris*. *Biochem. J.*, **145**: 397–400.

Tollefsen, D. M. 1992. The interaction of glycosaminoglycans with heparin cofactor II: structure and activity of a high-affinity dermatan sulfate hexasaccharide. **In**: *Heparin and related polysaccharides*. Lane, D. A., Björk, I., and Lindahl, U., Eds., Plenum Press, New York.

Torgerson, E. M., Brewer, L. C., and Thoma, J. A. 1979. Subsite mapping of enzymes. Use of subsite map to simulate complete time course of hydrolysis of a polymeric substrate. *Arch. Biochem. Biophys.*, **196**: 13–22.

Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. 1992. Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J. Biol. Chem.*, **267**: 10337–41.

Turnbull, J. E., and Gallagher, J. T. 1991a. Distribution of iduronate 2-sulphate residues in heparan sulphate. *Biochem. J.*, **273**: 553–59.

Turnbull, J. E., and Gallagher, J. T. 1991b. Sequence analysis of heparan sulphate indicates defined location of *N*-sulphated glucosamine and iduronate 2-ulphate residues proximal to the protein-linkage region. *Biochem. J.*, **277**: 297–303.

Tyrrell, D. J., Isihara, M., et al. 1993. Strucutre and affinity of a heparin-derived hexaccharide with high affinity for basic fibroblast growth factor. *J. Biol. Chem.*, **268**: 4684–89.

Varki, A. 1993. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*, **3**: 97–130.

Venkataraman, G., Sasisekharan, V., Cooney, C. L., Langer, R., and Sasisekharan, R. 1994. A stereochemical approach to pyranose ring flexibility: Its implications for the conformation of dermatan sulfate. *Proc. Natl. Acad. Sci., USA*, **91**: 6171–75.

Vesterberg, O. 1968. Studies on extracellular proteins from *Staphylococcus aureus*. III. Investigations on the heterogeneity of hyaluronate lyase using the method of isoelectric focusing. *Biochim. Biophys. Acta*, **168**: 218–27.

Vlodavsky, I., Eldor, A., et al. 1992. Expression of heparanase by platelets and circulating cells of the immune system: possible involvement in diapedesis and extravasation. *Invasion Metastasis*, **12**: 112–27.

Vlodavsky, I., Fuks, Z., et al. 1991. Extracellular matrix-resident basic fibroblast growth factor: Implication for the control of angiogenesis. *J. Cell. Biochem.*, **45**: 167–76.

Walenga, J. W., Hoppensteadt, D., and Fareed, J. 1991. Non-heparin glycosaminoclycan-derived drugs: A biochemical and pharmacologic perspective. *Sem. Thromb. Hem.*, **17 (suppl 1)**: 137–42.

Ward, O. P., and Moo-Young, M. 1989. Enzymatic degradation of cell wall and related plant polysaccharides. *CRC Critical Rev Biotechnology*, **8**: 237–74.

Wasteson, Å., Glimelius, B., Busch, C., Westermark, B., Heldin, C.-H., and Norling, B. 1977. Effect of a platelet endoglycosidase on cell surface associated heparan sulphate of human cultured endothelial and glial cells. *Thromb. Res.*, **11**: 309–21.

Wasteson, Å., and Westermark, B. 1976. Demonstration of a platelet enzyme degrading heparan sulphate. *FEBS Letters*, **64**: 218.

Winter, W. T., Taylor, M. G., Stevens, E. S., Morris, E. R., and Rees, D. A. 1986. *Biochem. Biophys. Res. Comm.*, **137**: 87–93.

Yamada, S., Yoshida, K., et al. 1993. Structural studies on the bacterial lyase-resistant tetrasaccharides derived from the antithrombin III-binding site porcine intestinal heparin. *J Biol Chem*, **268**: 4780–87.

Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. 1968. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J. Biol. Chem.*, **243**: 1523–35.

Yanagishita 1989. Metabolism of plasma membrane-associated heparan sulphate proteoglycans. **In**: *Heparin and related polysaccharides*. Lane, D. A., Bjork, I., and Lindahl, U., Eds., Plenum Press, New York.

Yang, V. C., Linhardt, R. J., Bernstein, H., Cooney, C. L., and Langer, R. 1985. Purification and characterization of heparinase from *Flavobacterium heparinum*. *J. Biol. Chem.*, **260**: 1849–57.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell*, **64**: 841–48.

Yoder, M. D., Keen, N. T., and Jurnak, F. 1993a. New domain motif: the structure of pectate lyase C, a secreted plant virulence factor. *Science*, **260**: 1503–07.

Yoder, M. D., Lietzke, S. E., and Jurnak, F. 1993b. Unusual structural features in the parallel b-helix in pectate lyases. *Structure*, **1**: 241–51.

Yost, H. J. 1992. Regulation of vertebrate left-right asymmetries by extracellular matrix. *Nature*, **357**: 158–61.

Zern, M. A., and Reid, L. M. 1993. *Extracellular Matrix*, Marcel Dekker, New York.

Zhang, J., Cousens, L. S., Barr, P. J., and Sprang, S. R. 1991. Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1β. *Proc. Natl. Acad. Sci., USA*, **88**: 3446–50.

Zhu, X., Hsu, B. T., and Rees, D. C. 1993. Structural studies of the binding of the anti-ulcer drug sucrose octasulfate to acidic fibroblast growth factor. *Structure*, **1**: 27–34.

Zhu, X., Komiya, H., et al. 1991. Three-dimensional structures of acidic and basic fibroblast growth factors. *Science*, **251**: 90–93.