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SEPARATION AND IDENTIFICATION OF GLYCOSAMINOGLYCANS

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INTRODUCTION

The term "mucopolysaccharides" was originally introduced by Meyer[1] to describe "hexosamine-containing heteropolysaccharides of animal origin occurring in a pure state or as protein salts". Many of the names originally assigned to the mucopolysaccharides have since been revised in an effort to systematize the nomenclature (Table 1). Jeanloz[2] in 1960 proposed the term "glycosaminoglycuronoglycans" in place of mucopolysaccharides as in most cases they are composed of amino sugars (glycosamino-) and uronic acids(glycurono-) joined in long chains(-glycans). For the sake of simplicity, the term "glycosaminoglycans" is getting acceptance in place of the rather lengthy term "glycosaminoglycuronoglycans" as well as the old and perhaps more familiar term "mucopolysaccharides". As a matter of convenience, the term "polysaccharides" will be frequently used here, instead of glycosaminoglycans.

Though connective tissues appear in different forms in various parts of the body, there is a fundamental similarity in their components. Three major components are generally recognized in connective tissues: the cells, the extracellular fibers, and the extracellular amorphous ground substance. The fibrillar elements and the ground substances , which are present in greater abundance, give connective tissues their main characteristics. The extra-

TABLE I

NOMENCLATURE AND COMMON ABBREVIATION FOR GLYCOSAMINOGLYCANS

New Terms		Old Terms	
Glycosaminoglycans	GAG	Acid mucopolysaccharides	AMPS
Hyaluronic acid	HA	Hyaluronic acid	HA
Chondroitin	Ch	Chondroitin	Ch
Chondroitin 4-sulfate	C-4-S	Chondroitin sulfate A	CSA
Chondroitin 6-sulfate	C-6-S	Chondroitin sulfate C	CSC
Dermatan sulfate	DS	Chondroitin sulfate B	CSB
Heparan sulfate	HS	Heparitin sulfate	HS
Heparin	Hep	Heparin	Hep
Keratan sulfate I*	KS-I	Corneal keratosulfate	KS
Keratan sulfate II*	KS-II	Skeletal keratosulfate	

* KS is not a GAG, but a glycoprotein with many properties similar to those of GAG.

cellular ground substances are composed of a variety of macromolecules. The glycosaminoglycans form an important part of the ground substances and occur bound to protein in a stable linkage, with the probable exception of hyaluronic acid. Though little is known about the structure of the polysaccharide-protein complexes, these macromolecules are generally referred as "proteoglycans". The relative proportion of carbohydrate and protein in connective tissue macromolecules may vary widely, from a preponderance of carbohydrate in proteoglycans to a preponderance of protein in glycoproteins. Therefore, it may be assumed that the proteoglycans might be a class of glycoproteins with especially extensive carbohydrate moieties. Many of the techniques employed in the studies of glycoproteins in general may also be applied in investigations of proteoglycans or glycosaminoglycans.

For the purpose of clarification, the term "glycosaminoglycans" is used for the macromolecules isolated from the proteolytic digests of connective tissues or after extraction with alkali. The glycos-

aminoglycans thus obtained are, in some degree, artefacts - the breakdown products of proteoglycans, in part free of peptides and in part still covalently linked peptide chains of variable length. The recent methodological advances have provided us with the better understanding of the role of glycosaminoglycans in health and disease.

SOME ASPECTS OF CHEMICAL STRUCTURE

The majority of glycosaminoglycans are composed of alternating units of a uronic acid (D-glucuronic acid or L-iduronic acid). and a hexosamine (D-glucosamine or D-galactosamine). The hexosamine is usually N-acetylated and an ester sulfate group may also be present at the 4 or 6 position of the hexosamine moiety. The resulting polymers assume linear configurations, although the possibility of branching cannot be excluded in certain instances. Each type of polysaccharide may be strictly classified by its hexosamine constituent as a glucosaminoglycan (hyaluronic acid, heparan sulfate, heparin, keratan sulfate) and a galactosaminoglycan (chondroitin, chondroitin 4- and 6-sulfates, dermatan sulfate), respectively. Hybrid forms containing both hexosamines so far have not been detected. The distribution of uronic acid residues is more complex. While some polysaccharides (hyaluronic acid, chondroitin, chondroitin 4- and 6-sulfates) have D-glucuronic acid as their sole uronic acid component, others (dermatan sulfate, heparan sulfate, heparin) occur as hybrids containing both L-iduronic acid and D-glucuronic acid residues in the same molecule. Polysaccharides containing L-iduronic acid exclusively have not been reported.

The similarity of the carbon skeleton of chondroitin 4- and 6-sulfates and dermatan sulfate is quite obvious (Fig. 1). In the first two, the uronic acid moiety is D-glucuronic acid. The glucuronidic linkage is β-1,3, and the galactosaminidic is β-1,4. Chondroitin 4- and 6-sulfates have identical repeating sugar units except for the position of the sulfate ester group. In dermatan sulfate, the uronidic moiety is L-iduronic acid (the 5 epimer of D-glucuronic acid) and the iduronidic linkage is α-1,3. The structures of heparin and heparan sulfate are not conclusively defined. It

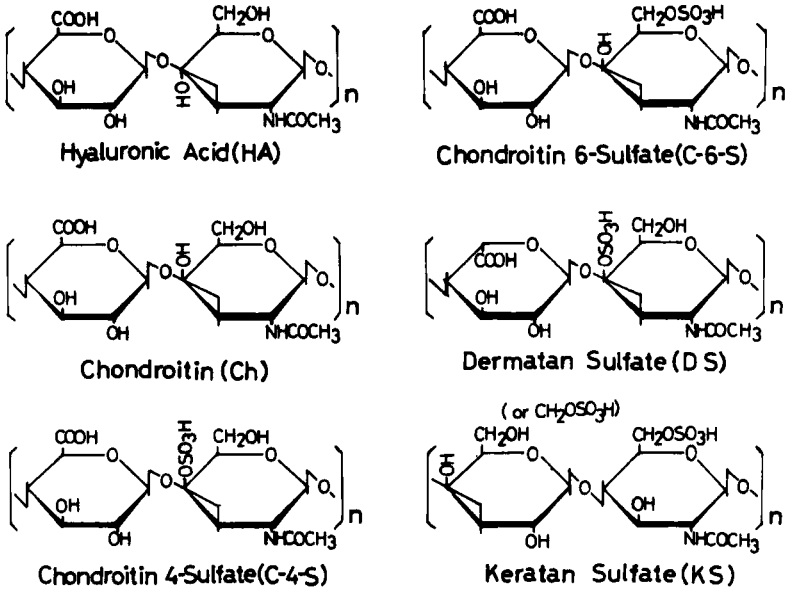

FIGURE 1. Structure of disaccharide repeating units of glycosaminoglycans. Heparin and heparan sulfate are not shown in this figure.

has been thought that the carbohydrate backbone of heparan sulfate seems to be similar to that of heparin, both having alternating units of D-glucuronic acid and D-glucosamine joined by α-1,4 linkages. These two polysaccharides differ with respect to sulfate and acetyl contents; heparan sulfate having more N-acetyl and fewer N- and O-sulfates than heparin. However, recent structural studies have shown that heparin and heparan sulfate contain, besides D-glucuronic acid residues, considerable amounts of L-iduronic acid moieties and their glycosidic linkages have both α- and β-configurations.[3-7] Unlike other glycosaminoglycans, keratan sulfate lacks uronic acid and consists,instead, of equimolar amounts of galactose and N-acetylglucosamine, linked by alternating β-1,4 and β-1,3 linkages. It is reasonable to assume that keratan sulfate is not a

glycosaminoglycan but a glycoprotein with many properties similar to those of glycosaminoglycans.

Although the major structural features of the glycosaminoglycans have been identified, these polymers, as isolated from the tissues, show a degree of heterogeneity. It is likely that the individual glycosaminoglycans are not single components but rather families of closely related substances. The heterogeneity of glycosaminoglycans manifests itself in several ways, including (a) molecular polydispersity of the polysaccharide chains; (b) variation in the degree of sulfation; and (c) the coexistence of both L-iduronic and D-glucuronic acid residues in the same chain of polysaccharides, such as heparin, heparan sulfate, and dermatan sulfate. The occurrence of the hybrid copolymers is demonstrated in various preparations of dermatan sulfate. For example, dermatan sulfates from pig skin and human umbilical cord are composed of three types of repeating units: glucuronosyl-N-acetylgalactosamine 4-sulfate (A type), iduronosyl-N-acetylgalactosamine 4-sulfate (B type), and glucuronosyl-N-acetylgalactosamine 6-sulfate (C type). Pig skin dermatan sulfate is composed almost exclusively of 4-sulfated units (AB copolymer), whereas dermatan sulfate from umbilical cord contains both 4-sulfated and 6-sulfated hexosamine moieties in the same chain (BC copolymer).[8,9]

Depending on the method of preparation, the glycosaminoglycans may display considerable polydispersity even though isolated from the same tissue. Hyaluronic acid preparation obtained after proteolytic digestion is of considerably lower molecular weight than material obtained after water or salt extraction. Chondroitin 4- and 6-sulfates occur together in most tissues in varying proportions. It has been undertermined whether both types of ester sulfate groups occur as a hybrid molecule in the same chain. Recently, Seno *et al.*[10] demonstrated the presence of a hybrid tetrasaccharide composed of a 4-sulfated and a 6-sulfated disaccharide units in the chondroitin sulfate from bovine tracheal cartilage.

By definition, the sulfate content of chondroitin sulfate attains one sulfate residue per disaccharide repeating unit. Most preparations of chondroitin sulfates, however, are somewhat deficient in sulfate, indicating that all hexosamines are not sulfated. Because of these "sulfate gaps", the possible existence of an unlimited number of dissimilar molecules may be envisaged. In addition, particular chondroitin sulfate isomers, such as chondroitin sulfate D from shark cartilage and chondroitin sulfate E from squid cartilage contain oversulfated disaccharide subunits.[11] The structure of these oversulfated disaccharides, which are identified in the chondroitinase digests, are shown in Fig. 2. Dermatan sulfate preparations from pig skin and bovine lung contain an oversulfated disaccharide as minor subunit, which is a derivative of 4-sulfated disaccharide bearing an extra-sulfate group on the uronic acid moiety, while dermatan sulfate from hog intestinal mucosa contains another oversulfated disaccharide bearing two sulfate residues at the positions 4 and 6 of a galactosamine moiety.[12] As mentioned above, heparin and heparan sulfate contain L-iduronic acid as well as D-glucuronic acid residues. Some of the iduronic acid moieties of these polymers seem to be sulfated.

Keratan sulfate appears to be exclusive to cornea, cartilage, and nucleus pulposus. Methylation studies suggest that keratan sulfate is partially branched and there is an excess of galactose over hexosamine, which are thought to be at the branch points.[13,14] In addition to the two sugar components of repeating period, several other monosaccharides are integral components of this polymer: fucose, mannose, sialic acid, and galactosamine. Fucose and sialic acid presumably occupy non-reducing terminal portion in side branchs, but their exact location and distribution is still unknown. Preliminary evidence suggests that mannose is located close to the carbohydrate-protein linkage, since the glycopeptides obtained after mild acid hydrolysis are considerably enriched in mannose.[15] Though keratan sulfate is a highly heterogeneous group of compounds, two main classes of keratan sulfate are currently

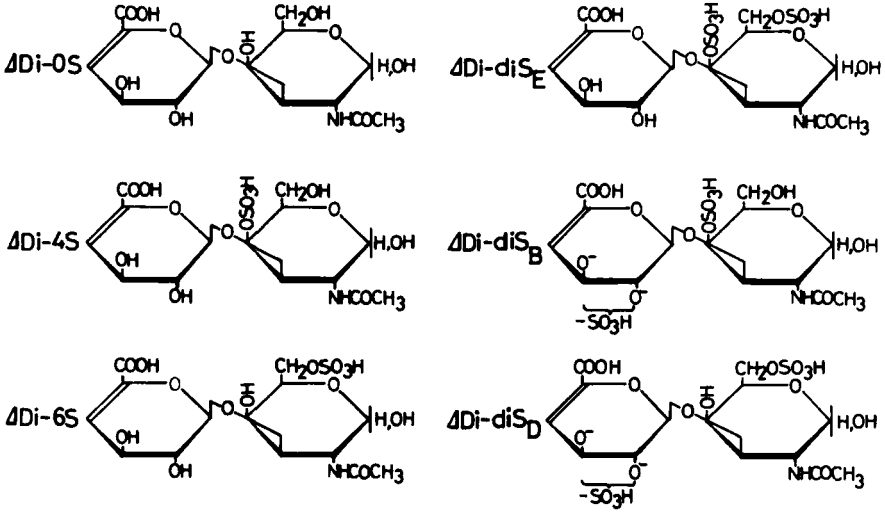

FIGURE 2. Structure of unsaturated disaccharides derived from chondroitin sulfates and dermatan sulfates after chondroitinase digestion. ΔDi-0S, 3-0-(β-D-$\Delta^{4,5}$-glucuronopyranosyl)-N-acetylgalactosamine; ΔDi-4S, 3-0-(β-D-$\Delta^{4,5}$-glucuronopyranosyl)-N-acetylgalactosamine 4-sulfate; ΔDi-6S, 3-0-(β-D-$\Delta^{4,5}$-glucuronopyranosyl)-N-acetylgalactosamine 6-sulfate; ΔDi-diS$_E$, 3-0-(β-D-$\Delta^{4,5}$-glucuronopyranosyl)-N-acetylgalactosamine 4,6-disulfate; ΔDi-diS$_B$, 3-0-(2 or 3-0-sulfo-β-D-$\Delta^{4,5}$-glucuronopyranosyl)-N-acetylgalactosamine 4-sulfate; and ΔDi-diS$_D$, 3-0-(2 or 3-0-sulfo-β-D-$\Delta^{4,5}$-glucuronopyranosyl)-N-acetylgalactosamine 6-sulfate. (from Suzuki et al., ref. 11, and Yamagata et al., ref. 90).

recognized; keratan sulfate I in cornea and keratan sulfate II in cartilage and nucleus pulposus.[16] The presence of galactosamine is limited to keratan sulfate II. The other sugars are found in varying proportions in both types of keratan sulfate. The sulfate residues are located mainly at the position 6 of the glucosamine moiety, but some of the galactose residues are also sulfated at the position 6 of the moiety. However, the degree of sulfation varied considerably with the tissue sources and the age of animals.

The structure of the polysaccharide-protein linkage regions has been established for several of common polysaccharides. The

structure of xylose-galactose-galactose sequence is found in chondroitin 4- and 6-sulfates, dermatan sulfate, heparin, and heparan sulfate (Fig. 3)[17-26] The xylose group is the potential reducing end of the polysaccharide chain and linked glycosidically to the hydroxyl group of serine. On the treatment with alkali, the xylosyl bond is broken by elimination reaction, in which seryl group of protein is converted to unsaturated dehydroalanine, and the total carbohydrate chain leaves the protein backbone with the xylosyl group at the reducing end. A glucuronic acid residue is linked β-1,3 to the above structure even in the case of dermatan sulfate in which the uronic acid is predominantly L-iduronic. N-acetylhexosamine is the next sugar residue, even in heparin, where most glucosamine is N-sulfated. In short, the linkage region of these polysaccharides is quite different from the rest of the chain. The linkage of corneal keratan sulfate is alkali stable and the polysaccharide chain is apparently linked to protein via the amide group of asparagine.[15,16,27,28] On the other hand, the alkali labile linkage of skeletal keratan sulfate is by N-acetylgalactosamine to threonine and serine.[13,28-30] These three types of the carbohydrate-protein linkage have been so far recognized in connective tissue glycosaminoglycans. Though the hyaluronic acid-proteoglycan interaction appears to be of fundamental importance for understanding how proteoglycans are organized in cartilage matrix, the exact structures of these binding regions remain to be elucidated.[31-33]

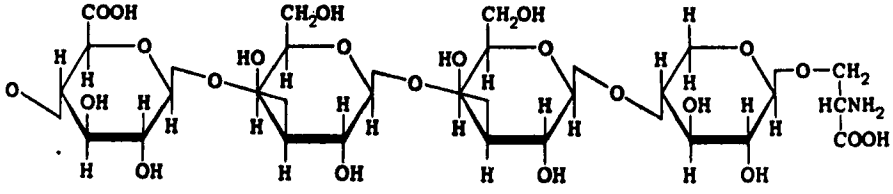

FIGURE 3. Structure of the carbohydrate-protein linkage region as established by Rodén and co-workers.

EXTRACTION OF GLYCOSAMINOGLYCANS FROM TISSUE

As the glycosaminoglycans occur bound to protein in the tissue, the principal problem encountered in the isolation of "pure" glycosaminoglycans concerns removal of bound protein under conditions which do not significantly degrade the polysaccharide chains. The only general methods which deserve attention are those that use proteolytic enzymes, alkali extraction or both. Chemical studies of glycosaminoglycans as a rule start with proteolytic digestion of the tissue, followed by the isolation and fractionation of glycosaminoglycans with a variety of procedures. In the next step, the purity of individual compounds has to be tested by chemical, physical and enzymatic methods.

Preliminary treatment

It is most important that solid tissues should be very finely ground by, e.g., Wylie mill, Potter homogenizer, or Waring blender in the cold. Since fat is an obstacle to the penetration of acid and neutral solutions into tissue, the tissues should be adequately defatted with acetone, acetone-ether, or chloroform-methanol mixtures. In order to facilitate the proteolytic digestion, finely ground and defatted tissue powder is heated in a boiling water bath for 30-60 min. This is intended to destroy mucolytic enzymes in the tissue and has the effect of denaturing protein, e.g., collagen which is resistant to some of the proteolytic enzymes in the native state. The denatured proteins are more easily susceptible to the proteolytic enzymes.

Extraction with water and salt solutions

It is well known that individual glycosaminoglycans may differ in their ease of extraction from connective tissue matrix. One of the connective tissue glycosaminoglycans, i.e., hyaluronic acid, may be obtained in reasonable yield from several tissues, such as human umbilical cord, vitreous body, and synovial fluid, by extraction with water or salt solutions. Chondroitin sulfate-proteoglycans may be extracted in good yield by high speed homogneization,[34] or by guanidine chloride extraction[35]; whereas

dermatan sulfate-proteoglycan remains in the residue after these extraction procedures and needs 6 M urea at 60° for its solubilization.[36,37] The extractability seems to be correlated inversely to the protein content of the proteoglycans. The easily extractable hyaluronic acid has a very low protein content, less than 1%, whereas the chondroitin sulfate-proteoglycans usually are reported to have a protein content of 10-20%. The dermatan sulfate-proteoglycan which is very difficult to extract, has a protein content as high as 50%.[37]

These extraction methods are of limited use if one wishes to extract quantitatively the entire polysaccharides from a given tissue. As the methods for proteoglycan preparation will not be described here in details, the reader are referred to recent discussions on this subject.[38,39]

Alkali extraction

Alkali treatment has been widely used in the past for the complete extraction of the tissue glycosaminoglycans. This procedure must be somewhat prolonged to give anything like a 100% liberation of polysaccharides. Usually, defatted and ground tissue is extracted with 0.5 M NaOH at 4° for 24 h with stirring. The extract is neutralized with acetic acid and deproteinized by a variety of methods. After dialysis against distilled water, the glycosaminoglycans are recovered by appropriate precipitation methods.

The basis of the release of glycosaminoglycans is a β-elimination reaction by which the carbohydrate-protein linkage is cleaved. Although many glycosaminoglycans are not markedly susceptible to alkali, long exposure at high pH results in some cleavages of glycosidic bonds. Because of incompleteness of extraction and considerable damage to polysaccharide chains, alkali extraction should be best confined to short treatment following proteolysis to remove residual protein where necessary. Although alkali treatment is now seldom used for preparative purposes to any extent, the alkali sensitivity of the xylose-serine linkage is a

valuable tool for the structural differentiation of the carbohydrate-protein linkage.

Proteolytic digestion

At present, alkali extraction of tissue has been largely superseded by proteolytic digestion. Ideally, it would be desirable to achieve proteolysis to the point where only amino acid remains attached to the polysaccharide but this is rarely feasible. In general, complete removal of the last few amino acids around the carbohydrate-protein linkage regions appears to be more difficult when a large carbohydrate chain is present than when the carbohydrate unit is relatively small. At present, digestion of tissue by a protease with broad specificity, such as papain or Pronase, is largely used prior to isolation of glycosaminoglycans. Extensive digestion of cartilage with papain yields a chondroitin sulfate preparation in which the residual peptides consist of five amino acids, on an average. Similarly, the product obtained after digestion with Pronase contains peptides with several amino acids. As papain is very resistant to temperature, it is possible to conduct the digestion at 65°-70°, with a great gain in speed and without bacterial growth. Skin, tendon, umbilical cord, nucleus pulposus, and cartilage may be almost completely solubilized by overnight digestion. Pronase digestion is usually carried out at 37°-40° and at pH 7.8-8.0 under a layer of toluene. After 24 h of incubation, half of the initial amount of Pronase is added to the mixture, and the digestion is continued until the pH of the digestion mixture remains unchanged. Pronase is more efficient than papain for cleaving the peptide bond in the carbohydrate-protein linkage, while papain is generally more efficient than Pronase in achieving solubilization of various tissues.

In some situations, pepsin and trypsin are used in sequence. The activity of pepsin is optimal at pH 1.5. Some sulfate groups, such as N-sulfated residues in heparin and heparan sulfate, are very sensitive to dilute acid. Since it is difficult to make a

reasonable allowance for any breakdown that may take place, the digestion with pepsin should be carried out with caution.

For certain purposes, particularly in studies of the structure of peptide sequence of various proteoglycans, it may be advantageous to use a protease which gives a more limited degradation. For example, proteolytic treatment of cartilage with trypsin or trypsin plus chymotrypsin yields "doublet" polymers in which two chondroitin sulfate chains are attached to the same peptide.[40,41] Further degradation of such doublet preparation by papain or alkali treatment makes it feasible to study the peptide backbone.

Corneal keratan sulfate may be relatively easily digested with proteolytic enzymes to yield a product which contains few amino acids and may be easily separated from the chondroitin and chondroitin 4-sulfate present in the tissue, whereas cartilage keratan sulfate is more resistant to proteolytic digestion and closely associated with chondroitin 6-sulfate.[28] It has been demonstrated that a doublet fraction, which is isolated by Pronase digestion from cartilage proteoglycan and contains both chains of keratan sulfate and chondroitin 6-sulfate on the same peptide, migrated as a single component in electrophoresis, but that cleavage of the carbohydrate-protein linkage with mild alkali subsequently permits the separation of the two polysaccharides. It may be conceived that cartilage proteoglycan contained keratan sulfate and chondroitin sulfate in the same protein backbone and after treatment with alkali, chondroitin sulfate is split off the protein core, whereas keratan sulfate remains covalently bound to the protein.

RECOVERY OF GLYCOSAMINOGLYCANS FROM PROTEOLYTIC DIGESTS

After proteolysis, it is generally advantageous to remove low molecular weight digestion products and small amounts of residual protein prior to isolation and fractionation of glycosaminoglycans. Contaminating protein is removed by treatment with kaolin and Lloyd's reagent or Sevag's method. But these procedures

are time-consuming and the repetition of the procedure results in a considerable loss of the products. More conveniently, deproteinization is accomplished by precipitation of protein with trichloroacetic acid. After addition of trichloroacetic acid to a final concentration of 10%, the mixture is kept at 0° for several hours and the resultant precipitate is removed by centrifugation. The supernatant liquid is neutralized and dialyzed against distilled water in the cold. The treatment with trichloroacetic acid should be carried out in the cold, because some sulfate groups are unstable at low pH range. Loss of low molecular weight glycosaminoglycans, such as heparin, heparan sulfate, and keratan sulfate, may occur during dialysis. This may be prevented in part by heating the dialysis tube at 85° for 3 days before use to reduce the pore size.[42] As alternatives to dialysis, ultrafiltration through a graded Diaflo membrane or gel filtration on a Sephadex column may be preferred.

Ethanol precipitation[43]

Although dialysis of the digestion products facilitates the subsequent fractionation of polysaccharides, it is not always necessary. Undoubtedly, the safest and simplest way to obtain glycosaminoglycans in a suitable state for further fractionation is to precipitate them directly by the addition of ethanol from the digestion products in the cold. Though sufficient concentration of salt is necessary for complete precipitation of polysaccharides, this is seldom a problem with tissue digests, which generally contain buffer salts. If inorganic salts are precipitated at the same time, they may be removed by the repeated cycle of dissolution and ethanol precipitation. If a salt-free solution of polysaccharide is mixed with ethanol, no precipitation occurs. Then, the polysaccharide is brought out of solution by the addition of sodium or potassium acetate. The acetates have an advantage over many other salts in that they are highly soluble in ethanol, and there is no risk of precipitating the salt even with a large excess of ethanol. It is convenient to add ethanol containing 1-5% of potassium or sodium acetate, or a 4:1 mixture of ethanol and saturated sodium acetate

gradually until precipitation is complete. Any of the connective tissue glycosaminoglycans will be precipitated completely by 4 volumes of ethanol in the presence of sufficient salt. In the course of purification of crude proteolytic digests, however, it is preferable not to exceed about two volumes of ethanol so as to avoid simultaneous precipitation of unwanted digestion products. Since keratan sulfate is quite soluble in relatively high concentrations of ethanol and may remain in the supernatant after precipitation with two volumes of ethanol, a large excess of ethanol should be used if the presence of keratan sulfate in the dogests is suspected.

The initial ethanol precipitate from crude tissue digests may often be difficult to dissolve. On repeated dissolution and precipitation from water or salt solutions, the interfering impurities are removed in part, and the precipitate thus becomes easier to dissolve. In order to ensure complete removal of salts, it may be preferable to repeat the process of dissolution and precipitation several times. Finally, glycosaminoglycans are recovered as sodium salts in the presence of sodium acetate by the addition of ethanol.

Precipitation with quaternary ammonium salts

An alternative to ethanol precipitation is the precipitation of glycosaminoglycans with quaternary ammonium salts, such as cetylpyridinium chloride (CPC) or cetyltrimethylammonium bromide (CTAB). The use of quaternary ammonium salts for this purpose has been reviewed at length by Scott.[44,45] The glycosaminoglycans form complexes with quaternary ammonium ions, which are insoluble in aqueous solutions of low ionic strength. Since CPC has a strong ultraviolet absorption at 260 nm, it is easily detectable if present in a final preparation. On the other hand, CTAB has no ultraviolet or visible light absorption and the information whether protein or nucleic acids have been efficiently removed is readily ascertained. CPC is now widely used for the isolation and fractionation of glycosaminoglycans. For complete recovery, the polysaccharide should be precipitated with sufficient amounts of CPC (usually, 3 mg of CPC per 1 mg of polysaccharide) at neutrality. The final mixture should

contain at least 0.05% of free CPC. The presence of some salts greatly facilitates the aggregation of the polysaccharide-CPC complexes, and a sodium chloride concentration of 0.03 M is usually satisfactory for this purpose. The sulfate ion is more effective than the chloride ion for the coagulation. If difficulties are encountered in obtaining a flocculent precipitate, sodium sulfate should be added to a final concentration of 0.02 M. As flocculation occurs more readily at elevated temperature, it is advantageous to keep the mixture at 37° for several hours before flocculation. However, for the precipitation of urinary glycosaminoglycans, it may be preferable to precipitate the glycosaminoglycans at 4° over a 24 h period while in the presence of 0.05-0.1% of CPC or CTAB.[46]

Keratan sulfate behaves in a different manner on precipitating with CPC. If CPC is added to keratan sulfate in 0.03 M NaCl, careful titration will yield a fine precipitate only at the equivalent point, but again, the precipitate is largely dissolved in an excess of CPC. Complete precipitation of keratan sulfate may be obtained if the polysaccharide is dissolved in 0.05 M borate buffer, pH 9.5, but this procedure is not specific for keratan sulfate, since some neutral polysaccharides may also be precipitated under the same condition. Therefore, complete recovery of keratan sulfate with CPC from tissue digests seems to be impractical.

Combination of polysaccharide and CPC occurs only at those pH's in which the polysaccharide is negatively charged. Hyaluronic acid is not precipitated below pH 2.0, where it is not ionized. Proteins are precipitable with quaternary ammonium salts under certain conditions but they are not precipitated at pH's lower than their isoelectric point. Since glycosaminoglycans are precipitated at pH's below the isoelectric points of the contaminating proteins, it is sometimes possible to precipitate the polysaccharides with CPC in the presence of large quantities of protein. If the protein has been adequately degraded by proteolytic enzymes or alkali, 90% or more of the proteolytic products are commonly left in the supernatant liquid during the precipitation of glycosaminoglycans with CPC.

In certain tissues with a high concentration of nucleic acids, these are often precipitated in the hyaluronic acid fraction. Much of the contaminating nucleic acids is usually eliminated during the deproteinization procedure with trichloroacetic acid. If nucleic acids are present in large excess over hyaluronic acid, it is preferable to subject the proteolytic digests to further treatment with nucleases prior to precipitation with CPC.

The polysaccharide-CPC complexes are collected, washed with 0.1% CPC, and dissolved in 2.0 M NaCl at 35°-40° or in 2.0 M NaCl containing 15% of ethanol to facilitate complete dissociation and solubilization of the complexes. The glycosaminoglycans are recovered by the addition of two volumes of ethanol or more in the case of the more soluble polysaccharides. This cycle is repeated several times to remove residual CPC completely and the polysaccharides are finally precipitated with ethanol in the presence of sodium acetate. Removal of quaternary ammonium ions can also be accomplished by precipitation with potassium thiocyanate.[42]

FRACTIONATION OF GLYCOSAMINOGLYCAN MIXTURE

Ethanol fractionation

Fractional precipitation with ethanol is one of the most suitable procedures for the large scale preparation of several polysaccharides. Fractionation with ethanol is most efficiently carried out in the presence of divalent metal ions, such as calcium,[47] barium,[48] and zinc.[49] The procedure recommended by Meyer *et al.*[47] has been applied with excellent results in many instances. Ethanol is added gradually to a 1-2% solution of polysaccharide mixture in 5% calcium acetate and 0.5 M acetic acid. The mixture is kept at 4° overnight and any precipitate formed is collected by centrifugation. Further precipitation with higher concentrations of ethanol is carried out in the same fashion. Dermatan sulfate precipitates in an ethanol concentration of 18-25%. Chondroitin 4-sulfate and chondroitin 6-sulfate precipitate at concentrations of 30-40% and 40-50% ethanol, respectively and with considerable overlap. As

hyaluronic acid also precipitates at about 30% ethanol, the separation between chondroitin sulfates and hyaluronic acid is not satisfactory. Keratan sulfate precipitates in 45-65% of ethanol. The exact concentrations of ethanol at which heparan sulfate and heparin may be precipitated, however, have not yet been reported. If several polysaccharides are present together, it may be more advantageous to fractionate the mixture into the groups with different charge density by the CPC method prior to ethanol fractionation.

The ethanol fractionation procedure is refined by the use of inert supporting media, such as cellulose, on which the polysaccharide mixture is precipitated with ethanol, followed by elution with stepwise decreasing concentrations of ethanol. A solution of polysaccharide mixture in 0.3% barium acetate is applied on a cellulose column which has been equilibrated with 80% ethanol containing 0.3% barium acetate. The column is then eluted with decreasing concentrations (80 → 20%) of ethanol containing 0.3% barium acetate. This procedure has been efficiently used by Gardell[48] for the isolation of keratan sulfate. The fraction from nucleus pulposus eluted with 35% ethanol contains glucosamine, 21.1%; galactosamine, 0.3%; galactose, 16.8%; fucose, 2.3%; and S, 5.45%.

In a recent application of this method to the study of dermatan sulfate preparations, it was concluded that the fractionation occurs almost exclusively on the basis of uronic acid composition, i.e., the relative proportions of iduronic acid and glucuronic acid, whereas molecular weight and sulfate content are of little importance.[50] Dermatan sulfate is , thus, separately precipitated with ethanol in the presence of chondroitin sulfates. Similarity in charge density makes it impossible to separate dermatan sulfate from chondroitin sulfates by the fractionation methods based on the difference in charge density of the polysaccharides.

In summary, ethanol fractionation of a polysaccharide mixture is one of the most suitable procedures for the isolation of dermatan sulfate and keratan sulfate.

As a matter of convenience, a copper precipitation method for the isolation of dermatan sulfate is described here.[51] Dermatan

sulfate is almost selectively precipitated with Benedict's reagent, leaving chondroitin sulfates, heparan sulfate and heparin in the supernatant. This method is simple to perform and yields reasonably pure preparations of dermatan sulfate. However, since this isolation procedure is carried out in a highly alkaline condition, some breakdown of polysaccharide chain may occur.

Fractionation with quaternary ammonium salts

The quaternary ammonium complexes of polysaccharides are dissociated and dissolved by inorganic salts at certain concentrations which depend largely on the charge density of the polymers. The critical salt concentration required to dissolve the complex of a given polysaccharide is well defined, reproducible, and characteristic of the polysaccharide. Since the glycosaminoglycans in mammalian tissues theoretically fall into three groups (based of their charge density), the differences in critical salt concentrations of their quaternary ammonium complexes permit the separation of glycosaminoglycans into three fractions: hyaluronic acid and chondroitin; chondroitin 4-and 6-sulfates, dermatan sulfate, and heparan sulfate; and heparin[44] (Table II). Keratan sulfate behaves in a different manner on CPC-precipitation and is not classified in either of these groups.

TABLE II
GROUP SEPARATION OF GLYCOSAMINOGLYCANS BASED ON CHARGE DENSITY
(Scott, ref. 44)

		Ionized anionic groups per sugar residue	Ratio of sulfate to carboxyl groups
Group I.	Hyaluronic acid, chondroitin	0.5	0
Group II.	Chonroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparan sulfate	1.0	1.0
Group III.	Heparin	1.5 - 2.0	2.0 - 3.0

In practice, fractionation based on the critical salt concentration has been carried out in three ways:[52]

(a) The polysaccharide and CPC are mixed in a salt concentration sufficiently elevated to prevent any of the polysaccharide from forming complexes. By dilution of the salt solution, the different polysaccharides are precipitated in order of decreasing critical salt concentration. With sodium chloride as the electrolyte, it is suitable to use an initial concentration of 2.0 M, and the solution is successively diluted to 1.4, 1.2, 0.5, and 0.1 M. The use of 0.05% CPC as diluent for each step is recommended. Heparin of high purity is precipitated at 1.4 M; material of lower anticoagulant activity and lower sulfate content, including some heparan sulfate, precipitates on further dilution to 1.2 M; the remaining heparan sulfate, chondroitin 4-and 6-sulfates, and dermatan sulfate precipitate on dilution to 0.5 M; and finally, hyaluronic acid is obtained on dilution to 0.1 M.[43]

(b) In simpler mixtures of known composition, the polysaccharide may be precipitated in a salt concentration sufficient to prevent precipitation of one group of components, while allowing others to precipitate completely. These are recovered by ethanol precipitation of the supernatant.

(c) All glycosaminoglycans are precipitated with CPC and the different complexes are then brought into solution by extracting the precipitate with solutions of increasing salt concentrations. The CPC-complex may not always dissolve completely at room temperature (in the presence of NaCl concentration sufficient to dissolve a polysaccharide-CPC complex), because the particles become coated with insoluble CPC which prevents further solvent action. On warming to 35°-40°, the complex dissolves in the saline solution with ease. The same effect is achieved by adding a small concentration of ethanol (15%) at room temperature. This phenomenon is attributed to the increase of critical solution temperature of CPC at higher salt concentration.[44] This difficulty is largely avoided if the

complex is deposited as a thin layer on an inert support such as cellulose.

The CPC-cellulose column procedure is used for preparative as well as microanalytical purposes.[52-54] The polysaccharide mixture is applied onto a cellulose column which has been equilibrated with 1% CPC, and after washing with 1% CPC, the precipitated compounds are fractionally eluted by stepwise increasing concentration of salts. With suitable series of eluents (Table III), the seven common polysaccharides may be largely separated from each other.[55] However, the change in chain length of the polymer gives rise to a marked alteration of elution pattern (Fig. 4).

Being soluble in excess of CPC, keratan sulfate appears in the first fraction from a CPC-cellulose column, eluted with 1% CPC. Since the impurities which are not precipitable will also be present in this fraction, this method cannot be used for the purification of keratan sulfate. In addition, a certain portion of the keratan sulfate is precipitated on the column and this fraction is only eluted at high salt concentration.

For several reasons, the fractionation of glycosaminoglycans with the use of quaternary ammonium salts is unfortunately not

TABLE III
ELUENTS USED IN THE FRACTIONATION OF GLYCOSAMINOGLYCANS ON CPC-CELLULOSE COLUMNS
(Svejcar and Robertson, ref. 55)

	Eluent	Polysaccharide eluted
1.	1% CPC	Keratan sulfate
2.	0.3 M NaCl in 0.05% CPC	Hyaluronic acid
3.	0.3 M $MgCl_2$ in 0.05% CPC	Heparan sulfate
4.	40% propanol-20% methanol-1.5% acetic acid in 0.05% CPC	Chondroitin 4-sulfate
5.	0.75 M $MgCl_2$-0.6% acetic acid in 0.05% CPC	Chondroitin 6-sulfate
6.	0.75 M $MgCl_2$ in 0.05% CPC	Dermatan sulfate
7.	1.25 M $MgCl_2$	Heparin

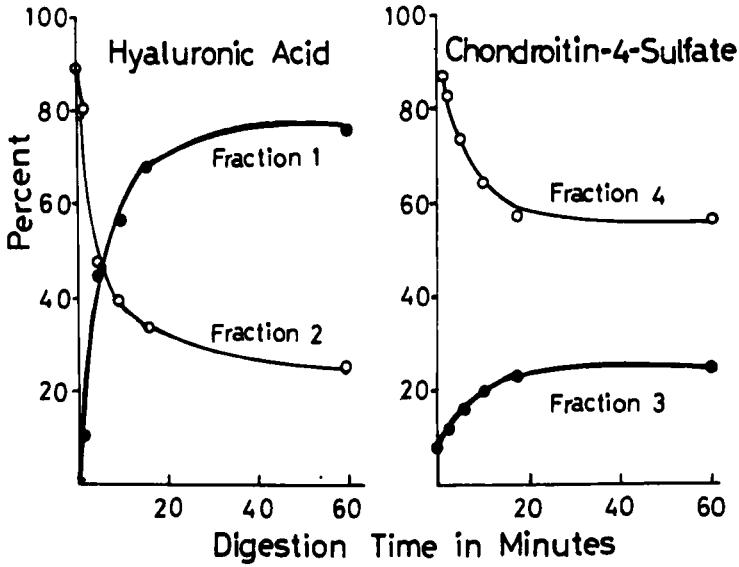

FIGURE 4. Effect of molecular weight on the fractionation of hyaluronic acid and chondroitin 4-sulfate: 1 mg of hyaluronidase (500 USP units) are added to 5 mg of glycosaminoglycan dissolved in acetate buffer, pH 5.0, and the solutions are incubated at 38°. Immediately, and at intervals thereafter, an aliquot is precipitated by addition of 3 vol of 5% potassium acetate in ethanol. The glycosaminoglycans are dissolved in 5% potassium acetate, precipitated with ethanol, redissolved, then fractionated. The ordinate is per cent of glycosaminoglycan fractionated. After 60 min incubation, all of the chondroitin 4-sulfate, but only 78% of the original hyaluronate, are still precipitated with ethanol. Fraction numbers are the same as in Table III. (from Svejcar and Robertson, ref. 55).

always as clear-cut as might be expected. The validity of the fractionation method with quaternary ammonium salts is markedly influenced by the heterogeneity of polysaccharide with respect to molecular size and charge density. It is well known that the critical salt concentration of the CPC complex of the same species of polymer is elevated with the increment of its molecular weight.[56] This is a major drawback of the procedure. Since it cannot be assumed that the polysaccharide mixture present in a particular

tissue digest will behave in the same way as the model compounds, it is necessary to determine the validity of the method in every single application.

Ion exchange chromatography

Several methods have been described for the separation of connective tissue glycosaminoglycans by ion exchange resins, such as Dowex 1-X2,[57] ECTEOLA-cellulose,[58] DEAE-cellulose,[59] DEAE-Sephadex,[60] and Deacidite FF.[61] It is not possible, however, to express a distinct preference for one ion exchange resin over the others, although Pearce et al.[62] has found Dowex 1 somewhat better than the cellulose exchangers with regard to sharpness of resolution. An example of a stepwise elution on a Dowex 1X2 (Cl^-) column is given in Fig. 5.[57] The separation of hyaluronic acid, heparan sulfate, chondroitin 4-sulfate, and heparin is accomplished by elution with 0.5, 1.25, 1.5, and 2.0 M NaCl, respectively. It is not possible, however, to obtain a clear-cut separation of each polysaccharide in all cases. The same considerations should be applied in the use of the ion exchange method that have been previously expressed regarding CPC-fractionation: namely, that it is necessary to establish the validity of the method in every application and the effect of the biological heterogeneity of the polysaccharides must be taken into account on the evaluation of the elution pattern.

In certain cases, this method has definite advantage over the CPC method.[43] Corneal keratan sulfate is obtained in high purity by Dowex 1-X2 column chromatography, since a large proportion of this polymer is strongly absorbed and requires 3-4 M NaCl for elution, whereas most other polysaccharides are desorbed at a lower salt concentration. Cartilage keratan sulfate, on the other hand, is eluted at a lower salt concentration with considerable overlap with chondroitin sulfate.

The recovery of polysaccharides from ion exchange resins is usually adequate, but sometimes a considerable proportion of the applied material may remain adsorbed even on elution with a high concentration of salts. This is especially true with preparation of hyaluronic acid.[62]

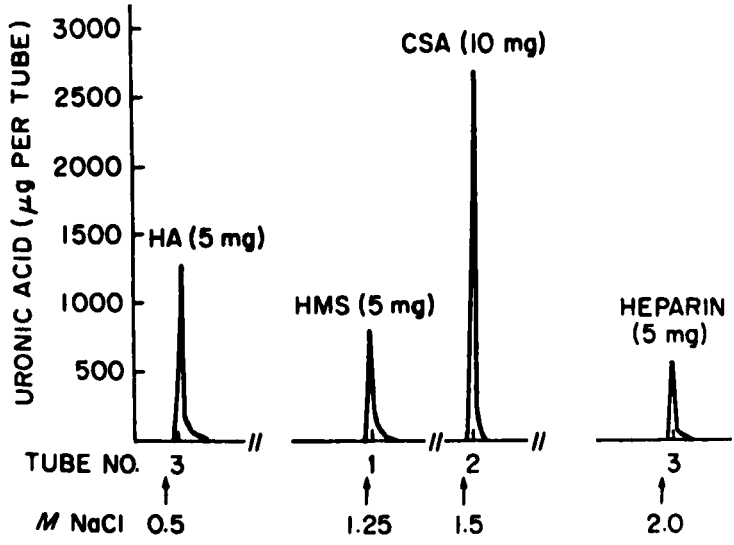

FIGURE 5. Elution diagram of hyaluronic acid (HA), heparan sulfate (HMS), chondroitin 4-sulfate (CSA), and heparin chromatographed individually on Dowex 1-chloride columns, 0.9 x 44 cm. Stepwise elution is used with NaCl concentrations of increasing molarity. The concentration of NaCl at which each of the substances is eluted is indicated as well as the tube number in which each substance appears in the effluent. (from Schiller et al., ref. 57).

Preparative electrophoresis

Electrophoretic separation of a polysaccharide mixture is rather widely used for analytical purposes. For preparative purposes, electrophoresis is carried out on a variety of supporting media, such as Celite,[63] Pevikon,[64,65] Agarose,[66] and so on. Wessler[65] reported that glycosaminoglycans have been separated by preparative electrophoresis on a Pevikon block in barium acetate into three fractions of increasing mobility: heparin-heparan sulfate; dermatan sulfate-hyaluronic acid; and the chondroitin sulfates. Though this procedure is suitable for group separation of the polysaccharides, it is of limited use for the preparation of glycosaminoglycans, probably owing to the limited loading capacity of the supporting media.

PREPARATION OF INDIVIDUAL GLYCOSAMINOGLYCANS

Basic principles used for isolation of individual glycosaminoglycans from tissue are outlined in the preceeding section. The marked heterogeneity of polysaccharides make it difficult to isolate separately a single polysaccharide species in the presence of the others. For the efficient isolation and purification of a particular polysaccharide, the tissue source must be chosen in which this polymer is one of the major components and is easily separated from the other polysaccharides therein. For instance, human umbilical cord is a convenient source for hyaluronic acid and chondroitin 6-sulfate. Chondroitin 4-sulfate and skeletal keratan sulfate(KS II) are prepared in good yield from bovine nasal septa. For the preparrative procedures of individual glycosaminoglycans, the readers are referred to an excellent review on this subject.[43]

CHEMICAL CHARACTERIZATION OF GLYCOSAMINOGLYCANS

Quantitative and qualitative analyses of the component sugars and other constituents often suffices to establish the tentative identity of a polysaccharide. Further analyses, including enzymatic digestion and physicochemical characterization may be carried out when a more complete picture of the properties of the product is required. These analyses are usually used for the evaluation of the isolated individual glycosaminoglycans. However, the analytical results also may provide a rough estimation of the proportion of each polysaccharide in a mixture. Estimations of uronic acid, hexosamine, and sulfate are essential for the identification of glycosaminoglycans, since these three components are the major constituents of the glycosaminoglycans.

Uronic acid

The most convenient procedures for preliminary characterization of the relative amounts of glucuronic acid and iduronic acid are the colorimetric methods which can be applied directly to the polysaccharide specimen without prior hydrolysis, particularly the

carbazole method and the orcinol method. With glucuronic acid as standard, the carbazole method gives the lower value for iduronic acid which is approximately 25% of the value for the same quantity of glucuronic acid; whereas the orcinol method gives nearly the same value for iduronic acid as for glucuronic acid. Theoretically, if a polysaccharide preparation is composed only of a gluccuronic acid-containing polymer, the estimated values in the carbazaole orcinol methods would be 1:1; whereas, if a preparation consists of only a iduronic acid-containing polymer, the carbazole to orcinol ratio of this polymer would be 0.25:1. Actually, it appears that the iduronic acid residues of dermatan sulfate behave roughly in the same fashion as the free ones in the carbazole and orcinol reactions. Used together, the carbazole and orcinol methods can therefore provide information concerning the relative proportions of glucuronic acid and iduronic acid in a mixture of dermatan sulfate and chondroitin sulfates.[67] When the carbazole to orcinol ratio is determined for a preparation of dermatan sulfate which contains almost exclusively iduronic acid residues, a value of close to 0.25 is observed.

In contrast to dermatan sulfate, heparin and heparan sulfate give a high color yield in the carbazole reaction despite the fact that more than half of the uronic acid residues of these polymers seem to be iduronic. In a mixture of heparin and heparan sulfate, the results of the carbazole reaction should be considered with caution.

Though precise quantitation of glucuronic acid and iduronic acid residues is essential for the identification and the structural analysis of many glycosaminoglycans, there is an inherent difficulty in the quantitative release of glucuronic acid and iduronic acid residues. Various hydrolysis conditions can be devised but the yields of glucuronic acid and iduronic acid are quite variable. Uronidic acid linkages are much more stable to acid hydrolysis than most other glycosidic bonds and, once liberated, the free uronic acid is partially destroyed before hydrolysis of the remaining

residues is complete. Considerably higher yield of the uronic acid residues is obtained by the procedure of Jaffrey and Rienits.[68] the yield of free glucuronic acid from chondroitin 4-sulfate is 74% by this procedure, but the yield of iduronic acid from dermatan sulfate is 50% by the same hydrolysis conditions. The liberated uronic acids are estimated by paper chromatography, ion exchange chromatography, or gas-liquid chromatography.

Hexosamine

Since there is no conclusive demonstration of hybrid polymers containing both glucosamine and galactosamine residues, qualitative and quantitative estimation of hexosamines is an indispensable part of the identification of glycosaminoglycans. Owing to the stability of uronidic linkages, the quantitative release of hexosamine from polysaccharide requires more drastic conditions than are commonly used in glycoprotein analyses. In general, glycosaminoglycans are hydrolyzed in a sealed tube in 3-4 M HCl for 15-16 h at 100°. Differential estimation of glucosamine and galactosamine is usually carried out by the ion exchange method using of a Dowex 50-X8 column [69] or by the amino acid analyzer.[70] The latter method is convenient and accurate. By the use of 15 x 0.9 cm column, glucosamine and galactosamine can be separately estimated by the use of an automatic amino acid analyzed in 60 minutes (Fig. 6).

Sulfate

Sulfate is completely liberated from polysaccharides by acid hydrolysis in 1 M HCl in a sealed tube held for at least 6 h at 100°. The inorganic sulfate is then precipitated as barium sulfate, which gives a stable turbidity in the presence of gelatin and can be determined spectrophotometrically.[71] N-sulfated groups may be determined after treatment of polysaccharide with nitrous acid.[72,73] A number of different procedures, which involve rate of acid hydrolysis, infrared spectra, and susceptibility to specific enzymes, are available for identification of the site of attachment of O-sulfate groups.

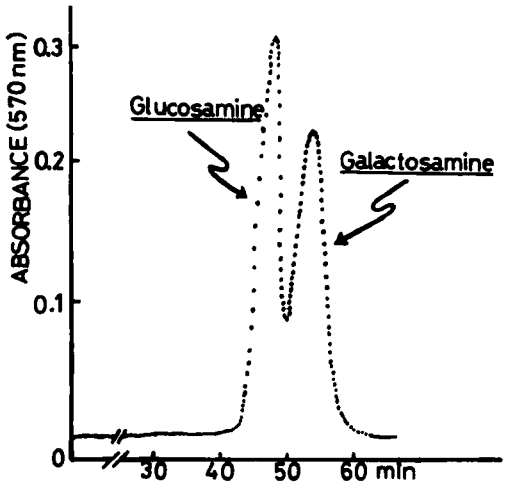

FIGURE 6. D-glucosamine·HCl and D-galactosamine·HCl, 0.2 µmoles each, are applied on an Amberlite CG-120, type III, column, 0.9 x 15 cm of a Hitachi KLA 3B amino acid analyzer. Elution is carried out with an ordinary 0.35 M NaOH-citrate buffer, pH 5.28, at 55°. Hexosamines can be separately estimated within 60 min, even in the presence of other amino acids.

SEPARATION OF SMALL QUANTITIES OF GLYCOSAMINOGLYCANS

Microcolumn method

Many procedures for the separation and isolation of individual polysaccharides are designed for preparative rather than for analytical purposes. When used for analytical work, they require large quantities of materials and cannot be adequately scaled down. The microcolumn of CPC-cellulose,[54,55] or ion exchange resins[62] are frequently used for the fractionation of small quantities of polysaccharides. Tanaka and Gore[74] devised a two-step method using a cellulose column. In the first step, CPC-polysaccharide complexes are eluted with 0.35 M and 0.65 M NaCl and in the second, the remaining CPC-polysaccharide complexes are disrupted with absolute ethanol

and 90% ethanol saturated with sodium acetate, followed by elution with decreasing concentrations of ethanol. This method may permit the sharp separation of individual components in human aortic polysaccharides. However, it is not always possible to obtain clear-cut separations of individual glycosaminoglycans by these methods, since they suffer from the same drawbacks that are inherent in the ordinary separation methods.

Thin-layer chromatography

Separation of individual glycosaminoglycans has been occasionally carried out by chromatography on silicated glass filter paper[75] and ordinally filter paper.[76] But the heterogeneity of the polymers makes it difficult to identify one polysaccharide in the presence of the others. Recently, Marzullo and Lash[77] reported that several common glycosaminoglycans can be separated from each other by thin-layer chromatography on plain Silica gel by a solvent system of n-propanol-concentrated ammonia-water (40:60:5, v/v). By this method, 1 μg amounts of glycosaminoglycans can be readily separated and visualized after staining with alcian blue.

Electrophoresis on cellulose acetate membrane

A large number of methods for the fractionation of glycosaminoglycans possess difficulties in separating different species of glycosaminoglycans. These difficulties are due to their marked heterogeneity with respect to molecular size and charge, and to the presence of hybrid structures. The effect of variations in molecular weight can be essentially overcome by using electrophoresis. On the other hand, variations in sulfate content greatly influence the migration rate in buffers ordinarily used for polysaccharide separation, such as sodium barbital, sodium or potassium phosphate, pyridine-formic acid and so on.

Recently, it has been found that electrophoresis on cellulose acetate membranes in specified buffers provides a rapid and sensitive method for identifying most of the tissue glycosaminoglycans. Haruki and Kirk[78] introduced the use of zinc salts as media for electrophoresis. Electrophoresis in zinc acetate or zinc sulfate

separates dermatan sulfate from the chondroitin sulfates, but does not differentiate dermatan sulfate from heparan sulfate. Electrophoresis in copper acetate of pH 3.5, differentiates hyaluronic acid, heparan sulfate, dermatan sulfate and the chondroitin sulfates from each other, but does not separate chondroitin 4-sulfate from chondroitin 6-sulfate.[79] Wessler[65] reported that barium acetate permits the separation of glycosaminoglycans into three groups: heparan sulfate-heparin; dermatan sulfate-hyaluronic acid; and the chondroitin sulfates. Chondroitin 6-sulfate migrates slightly faster than chondroitin 4-sulfate in this buffer, but the separation of these two polymers is incomplete. The separation of dermatan sulfate from the chondroitin sulfates is possible by the use of the salt solutions containing divalent metal ions as electrophoretic media, such as zinc acetate, copper acetate, cadmium acetate, cobalt acetate, barium acetate, calcium acetate, and so on. On the other hand, salt solutions containg mono- and trivalent cations do not separate these polymers.[80]

Calcium acetate gives a better separation between chondroitin 4-sulfate and chondroitin 6-sulfate. Hyaluronic acid, dermatan sulfate, chondroitin 4- and 6-sulfates are separated from each other in this buffer.[80,81] But it is desirable that heparin and keratan sulfate, which migrate close to the chondroitin sulfates in calcium acetate, be removed previously by ethanol fractionation or column chromatography.

A hybrid chondroitin sulfate containing both 4-sulfated and 6-sulfated repeating units in the same molecules, which is recently isolated from bovine tracheal cartilage, migrates as a single spot between chondroitin 4- and 6-sulfates, indicating the hybrid nature of this polymer.[10] Chondroitin sulfate D (ChS-D with an S:N ratio of 1.23) from shark cartilage,[82] which is oversulfated chondroitin 6-sulfate and contains an extrasulfate residue on the glucuronic acid moiety, migrated at the same rate as the chondroitin 6-sulfate marker. Oversulfated dermatan sulfate (SChS-B with an S:N ratio of 1.6) from shark skin,[83] which is a derivative of dermatan sulfate

bearing an extrasulfate group on the iduronic acid moiety, migrates at the same rate as standard dermatan sulfate with an S:N ratio of 1.0. On the other hand, chondroitin sulfate E (ChS-E with an S:N ratio of 1.56) from squid cartilage,[84] which contains 4,6-disulfated galactosamine moieties, migrated ahead of chondroitin 6-sulfate. Chondroitin sulfate H (ChS-H with an S:N ratio of 1.86) from hagfish notochord,[85] of which a repeating unit is composed of 4,6-disulfated galactosamine and iduronic acid, migrates faster than the dermatan sulfate marker in calcium acetate.[80] These results suggest that the order of mobility in calcium acetate depends more on the differences in the backbone structure and in the degree of sulfation on the galactosamine moiety, whereas the total sulfate content and the sulfation of uronic acid residues appear to be of less importance (Fig. 7).[65,80]

Total sulfate content of glycosaminoglycans is easily estimated by electrophoresis in 0.1 M HCl.[86] In 0.1 M HCl, the carboxyl group of polysaccharides is not dissociated so that the migration rate depends exclusively on sulfate content.

Since relative mobilities of various glycosaminoglycans differ in different electrolyte solutions, it is apparent that a combination of two electrophoretic systems may be essential for the conclusive identification of individual glycosaminoglycans on a small scale. Hata and Nagai[87] reported that two-dimensional electrophoresis on a cellulose acetate membrane with 0.1 M pyridin-0.47 M formic acid buffer of pH 3 in the first run and 0.1 M barium acetate of pH 8 in the second, permits the separation of the seven glycosaminoglycans: hyaluronic acid, keratan sulfate, heparan sulfate, heparin, dermatan sulfate, chondroitin 4- and 6-sulfates. As little as 0.5 μg of polysaccharides can be detected on the electropherogram after staining with alcian blue (Fig. 8).

After staining with alcian blue, the amounts of each polysaccharide are estimated by direct scanning densitometry of the corresponding spots[80] or after extraction of the dye bound to individual glycosaminoglycans.[88] Less than 1 μg of polysaccharide can be determined by these methods with an error of about 10%.

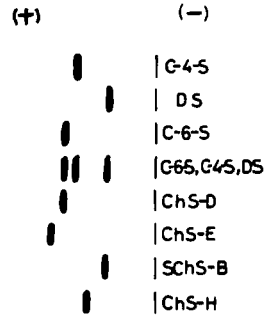

FIGURE 7. Electropherogram of oversulfated chondroitin sulfates compared with C-4-S, DS, and C-6-S on cellulose acetate strip in 0.3 M calcium acetate at 1.0 mA/cm for 3 h. ChS-D with S:N ratio of 1.23; ChS-E with S:N ratio of 1.56; SChS-B with S:N ratio of 1.60; and ChS-H with S:N ratio of 1.86. For further details, see the text. (from Seno et al., ref. 80).

Enzymatic estimation of chondroitin sulfate isomers

Saito et al.[89] devised the micromethods for the specific and sensitive measurement of chondroitin sulfate isomers. By the combined use of chondroitinases and chondrosulfatases, as little as 3 μg of each isomer can be estimated separately in the presence of the other glycosaminoglycans. The methodology and the specificity of the enzymatic procedure will be discussed in the next section.

SPECIFIC ENZYMES AVAILABLE FOR STRUCTURAL ANALYSES

The enzymatic method is an important part of the analytical process for the identification of connective tissue glycosaminoglycans. Testicular hyaluronidase had long been used for analytical as well as preparative purposes. In addition, several enzymes with the unusual property of degrading polysaccharides by an elimination reaction rather than by the usual hydrolytic pathway, are obtained

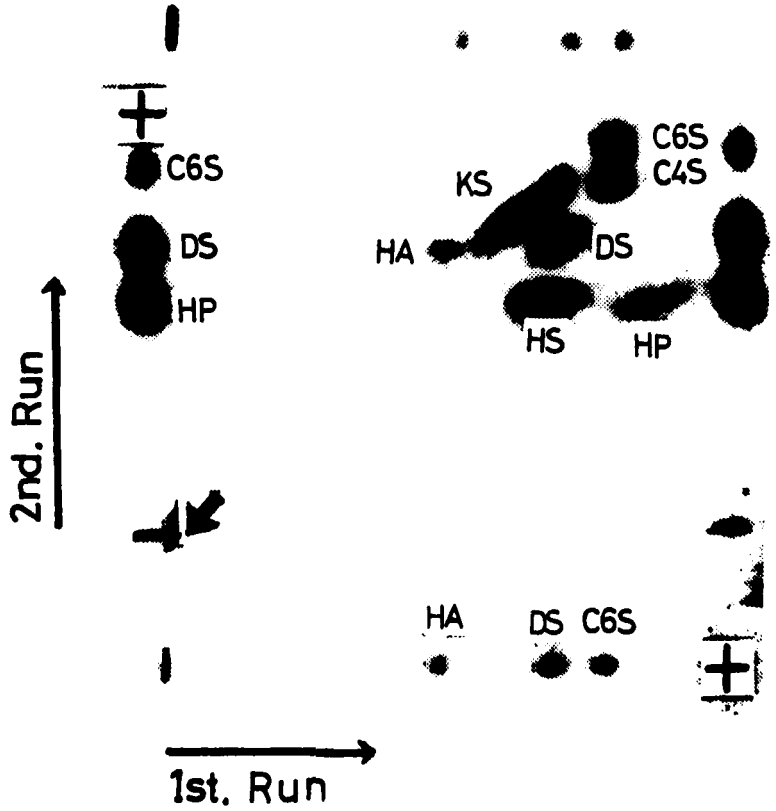

FIGURE 8. Two-dimensional electrophoresis of a mixture of seven reference glycosaminoglycans on cellulose acetate strip. Migration directions are marked with large arrows. Electrophoretic systems; 0.1 M pyridine/0.47 M formic acid, pH 3, at 1 mA/cm for 1.7 h in the first dimension and 0.1 M barium actate, pH 8, at 1 mA/cm for 4.5 h in the second. Stained with 0.1% Alcian blue in 0.1% acetic acid. (from Hata and Nagai, ref. 87).

from microorganisms. The commercially available products of bacterial chondroitinases and chondrosulfatases have provided a new set of valuable enzymatic tools for the quantitative and qualitative analyses of chondroitin sulfate isomers.[89,90] Recently, other two types of chondroitinases; chondroitinase C, acting specifically on

chondroitin 6-sulfate and chondroitinase B, acting specifically on dermatan sulfate,are isolated from extracts of adapted Flavobacterium heparinum.[91,92] Recently, the enzyme complex of heparin-induced flavobacteria was fractionated and purified. Two types of enzymes are isolated; one of them degrades heparin by elimination reaction, yielding unsaturated uronides and the other attacks specifically heparan sulfate in the same manner.[93,94] Further purification of these enzymes make it possible to degrade specific substrates and to analyze subunit structures of individual glycosaminoglycans.

Hyaluronidase

Several types of hyaluronidases are isolated from various sources (Table IV). Among them, testicular hyaluronidase is most widely used for the identification of hyaluronic acid, chondroitin, chondroitin 4- and 6-sulfates. However, it is limited to these substrates, and, because it reshuffles oligosaccharides by transglycosylation, it cannot be used for sequence studies. It has been demonstrated that, though L-iduronic acid is identified as the main uronic acid component of dermatan sulfate, a small amount of D-glucuronic acid is almost invariably present in preparations of dermatan sulfate. If a dermatan sulfate has a hybrid molecule containing both L-iduronic acid and D-glucuronic acid residues as an integral part of the carbohydrate chain, the presence of β-hexosaminidic linkage to D-glucuronic acid might be expected to render this polymer partially susceptible to testicular hyaluronidase. This assumption is verified with various dermatan sulfate preparations, demonstrating the existence of hybrid oligosaccharides containing both L-iduronic acd and D-glucuronic acid residues in the hyaluronidase-digests of dermatan sulfate preparations.[8,9]

A novel bacterial hyaluronidase is isolated and purified from Streptomyces hyalurolyticus nov. sp.[95] Streptomyces nyaluronidase attacks only hyaluronic acid, yielding unsaturated tetra- and hexasaccharides by elimination reaction. The other polysaccharides including chondroitin, are not susceptible to this

TABLE IV
CLASSIFICATION OF HYALURONIDASES

Source	Substrates	Mode of action	Main products
Testicular	HA, Ch, C-4-S, C-6-S, (DS)[+]	Endo-β-hexosaminidase, transglycosylation	U-H-U-H*
Snake venom	HA, Ch	Endo-β-hexosaminidase, transglycosylation	U-H-U-H*
Bacterial	HA, Ch	Endo-β-hexosaminidase by elimination reaction[‡]	ΔU-H*
Streptomyces	HA only	Endo-β-hexosaminidase by elimination reaction	ΔU-H-U-H* and ΔU-H-U-H-U-H*
Leech	HA only	Endo-β-glucuronidase	H-U-H-U*

[+] Dermatan sulfate is split only at few sites containing D-glucuronic acid groups; iduronosyl sites are resistant.

[‡] β-elimination by migration of H from C-5 of the glucuronosyl group to the O-atom of the hexosaminidic bond, thus leading to splitting of the bond with formation of compounds with $\Delta^{4,5}$-unsaturate glucuronosyl 1,3 N-acetylhexosamine.

An asterik(*) indicates the reducing terminal group.

Abbreviations: HA, hyaluronic acid; Ch, chondroitin; C-4-S and C-6-S, chondroitin 4- and 6-sulfates; DS, dermatan sulfate; U, glucuronic acid; and H, hexosamine.

enzyme. This enzyme is now available commercially and provides a useful tool for the identification of hyaluronic acid.

The mode of action of various hyaluronidases is summarized in Fig. 9.

Chondroitinase and chondrosulfatase(Fig. 10)

The term "chondroitinase" was introduced by Dodgson and Lloyd[96] to describe an enzyme from Proteus vulgaris NCTT 4636 that converts chondroitin sulfate to a sulfated disaccharide. A somewhat similar enzyme also has been found in Flavobacterium heparinum ATCC 13125.[97] Yamagata et al.[90] purified chondroitinase ABC and chondroitinase AC from extracts of adapted Proteus vulgaris and of Flavo-

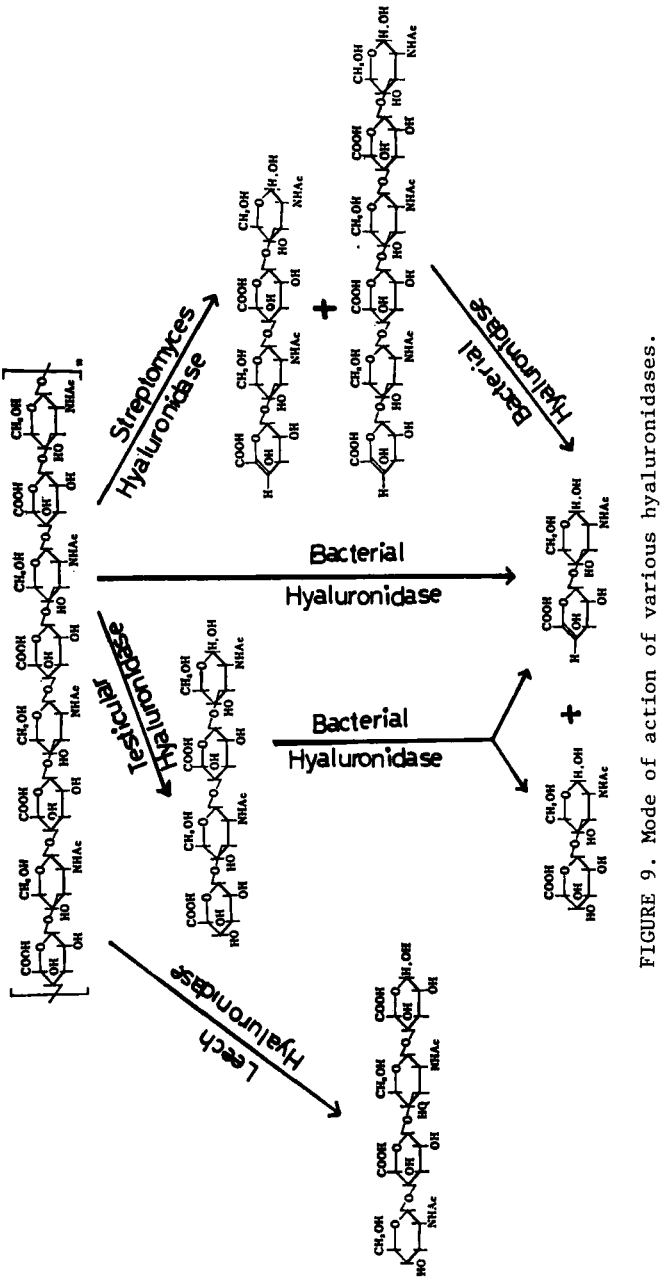

FIGURE 9. Mode of action of various hyaluronidases.

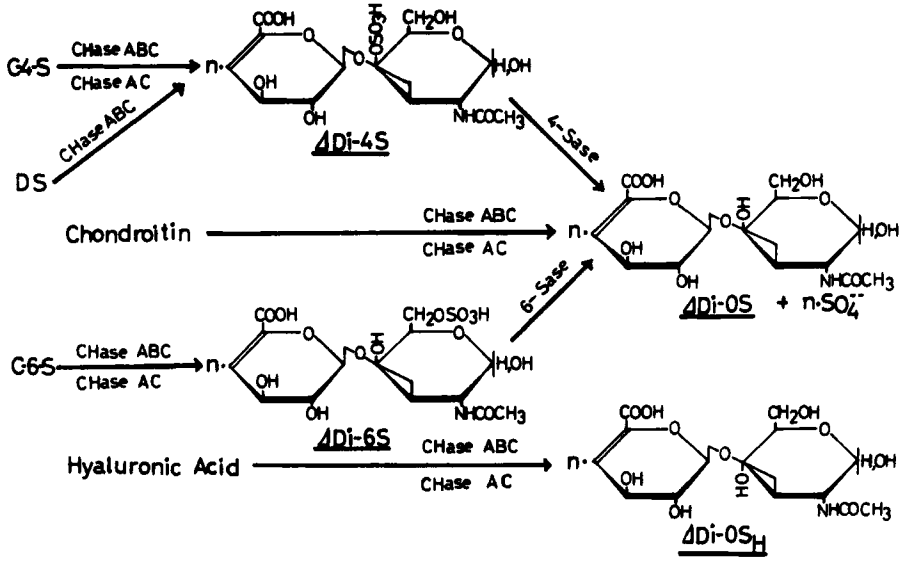

FIGURE 10. Reactions catalyzed by chondroitinases and chondrosulfatases. CHase AC, chondroitinase AC; CHase ABC, chondroitinase ABC; 4-Sase, chondro-4-sulfatase; 6-Sase, chondro-6-sulfatase; and ΔDi-0S$_H$, 3-O-(β-D-$\Delta^{4,5}$-glucuronopyranosyl)-N-acetylglucosamine. Further abbreviation are the same as in Fig. 2. (from Suzuki and co-workers, ref. 89 and 90).

bacterium heparinum, respectively. It has been conclusively demonstrated that the digestion products with these enzymes are $\Delta^{4,5}$-unsaturated disaccharides. Recently, chondroitinase AC-II, an enzyme of chondroitinase AC type, has been isolated and purified from extracts of Arthrobacter aurescens.[98] The activity and the mode of action of chondroitinase AC-II are identical with those of chondroitinase AC. The principal advantage of chondroitinase AC II is the simplicity of the purification procedure.

Chondroitinase ABC acts with almost equal facility on chondroitin 4- and 6-sulfates as well as on dermatan sulfate and at a reduced rate on hyaluronic acid. The specificity of the enzyme does not involve recognition of a particular size or a particular sulfate content of the substrate molecule. Oversulfated chondroitin

sulfates, such as chondroitin sulfate D from shark cartilage and chondroitin sulfate E from squid cartilage, are degraded in a similar manner, yielding unsaturated disulfated disaccharide units. Keratan sulfate, heparin, and heparan sulfate are not susceptible to this enzyme. Chondroitinase AC differs from chondroitinase ABC in that it does not degrade dermatan sulfate. However, D-glucuronic acid-containing hybrid sections in dermatan sulfate chains serve as substrates for chondroitinase AC. Subsequent characterization of the hybrid oligosaccharides containing both L-iduronic acid and D-glucuronic acid residues in the chondroitinase AC-digests permits the elucidation of hybrid structures in a given dermatan sulfate preparation.

Yamagata et al.[90] also have purified two types of chondrosulfatases, chondro-4-sulfatase and chondro-6-sulfatase, from extracts of Proteus vulgaris. Chondro-4-sulfatase catalyzes the convertion of unsaturated and saturated 4-sulfated disaccharides to the corresponding non-sulfated disaccharides and SO_4^{2+}, but does not attack 6-sulfated disaccharide. In contrast, chondro-6-sulfatase carries out the desulfation of the disaccharide 6-sulfate, but does not attack the disaccharide 4-sulfate isomer. It has been thought that chondrosulfatases hydrolyze ester sulfates of the disaccharides, but not of higher oligosaccharides. However, the results of a recent work indicated that chondro-4-sulfatase attacks the C-4 ester sulfate at the reducing end of the sulfated tetrasaccharides, which are prepared from chondroitin sulfate after the hyaluronidase digestion, regardless of the position of ester sulfate in the non-reducing disaccharide residue. On the other hand, chondro-6-sulfatase does not attack the C-6 ester sulfate of sulfated tetrasaccharides.[99]

(a) Separation of chondroitinase digests with disaccharide mapping

The use of chondroitinases and chondrosulfatases permits the precise differential estimation of small quantities of chondroitin sulfate isomers. Three principal procedures are devised by Saito et al.[89] Among these, only the disaccharide mapping method is described here. Equal aliquots of a test sample are separately

subjected to the digestion with chondroitinase AC and ABC. The digestion products are separated by paper chromatography by a solvent system of 1-butanol-acetic acid-1 M ammonia (2:3:1, v/v) or n-butyric acid-0.5 M ammonia (5:3, v/v) with descending technique. With this method, ⊿Di-6S, ⊿Di-4S, and ⊿Di-0S are separated from each other. ⊿Di-6S, ⊿Di-4S, and ⊿Di-0S are the repeating units of chondroitin 6-sulfate, chondroitin 4-sulfate, and chondroitin, respectively(see Fig. 10). After drying, the unsaturated disaccharides are located by viewing under ultraviolet light of short wavelength (Fig. 11). The regions containing unsaturated disaccharides are cut out and extracted with 0.01 M HCl at 50° for 10 min. The absorbance of the extracts is measured at 232 nm against appropriate blanks. As molar absorption coefficients of ⊿Di-6S, ⊿Di-4S, and ⊿Di-0S are 5500, 5100, and 5700, respectively, the amounts of each unsaturated disaccharide can be calculated from the absorption measurements.

The amounts of ⊿Di-4S in chondroitinase ABC system are the sum of unsaturated disaccharides derived from chondroitin 4-sulfate and dermatan sulfate, whereas ⊿Di-4S in chondroitinase AC system is derived only from chondroitin 4-sulfate. ⊿Di-6S in both systems is derived from chondroitin 6-sulfate. Thus, the amount of each polymer is calculated from the results of disaccharide subunit assay. Assuming that sodium chondroitin sulfate is composed of such repeating units as $C_{14}H_{19}NSO_{14}Na_2$ (period weight, 503), the weight of sodium chondroitin sulfate is calculated from the results multiplying by 503. The common occurrence of relatively small amounts of ⊿Di-0S usually indicates the presence of undersulfated isomers, but not chondroitin itself.

Although this method requires relatively larger amounts of the specimen than the other assay methods, it is indispensable for the structural analysis of chondroitin sulfate isomers. With this method, novel structural subunits are identified in various preparations of isomeric chondroitin sulfates.

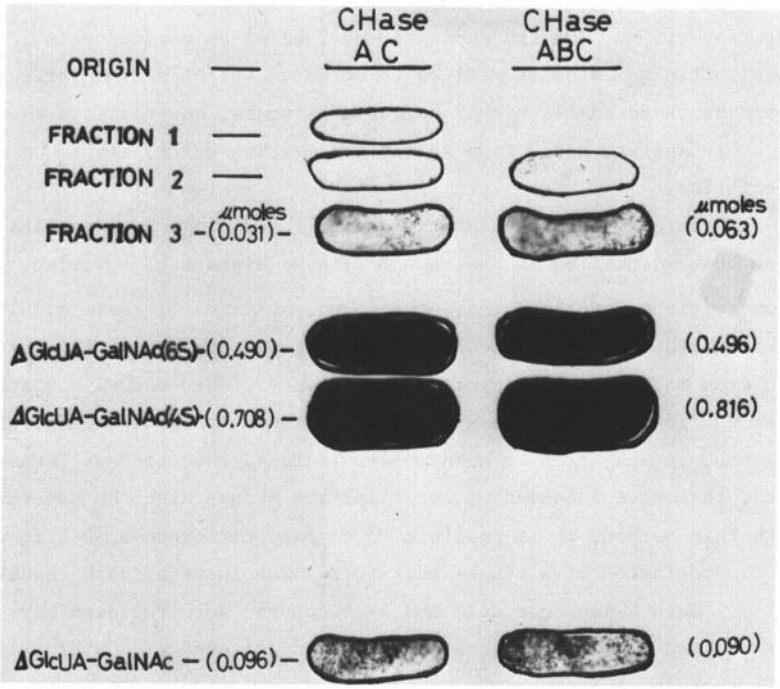

FIGURE 11. Paper chromatographic separation of chondroitinase digests of urinary glycosaminoglycans. The condition for enzymatic digestion is the same as in ref. 89. The first development is carried out in 1-butanol-acetic acid-water (2:1:1, v/v), followed by the second in 1-butanol-acetic acid-1 M ammonia (2:1:1, v/v) with descending technique for 18 h in each occasion. Tracing of ultraviolet absorbing spots are represented. This figure is represented as a negative print; blach and white are reversed in the actual chromatogram. The amounts of $\Delta^{4,5}$-glucuronic acid residues (per mg of urinary glycosaminoglycans) are indicated in parentheses. CHase AC, chondroitinase AC-digests; CHase ABC, chondroitinase ABC-digests. Faint ultraviolet-absorbing materials, Fraction 1-3, are also demonstrated in the digests.

(b) Heterogeneity in the degree of sulfation

Most preparations of chondroitin sulfate are somewhat deficient in sulfate, indicating that some hexosamine moieties are not sulfated. On digestion with chondroitinase, sulfated and non-sulfated disaccharides are derived from these preparations in vary-

ing proportions. On the contrary, oversulfation may occur in chondroitin sulfates as well as in dermatan sulfates from various sources. Even in the highly sulfated polymers, however, the existence of "sulfate gaps" (non-sulfated repeating units) may be identified.

Three types of unsaturated disulfated disaccharide units have been identified in the chondroitinase digests of a variety of chondroitin polysulfate and dermatan polysulfate.[11] These disulfated disaccharides are separated from nonsulfated and monosulfated isomers and from each other by paper chromatography. When each disulfated disaccharide, which is separated and purified from the chondroitinase digests, is subjected to chondro-4-sulfatase, chondro-6-sulfatase,or both, the exact location of ester sulfate groups might be determined. With this method, it is possible to compare differences as slight as the variation of a single sulfate residue in each 15-20 repeating units. Both L-iduronic acid and D-glucuronic acid residues give rise to the same $\Delta^{4,5}$-glucuronic acid if attacked by chondroitinase ABC, whereas if attacked by chondroitinase AC, $\Delta^{4,5}$-glucuronic acid is liberated only from D-glucuronic acid residue, but not from L-iduronic. By the use of both digestion systems, the nature of the uronic acid in parent polysaccharide chain might be elucidated. For example, in addition to ΔDi-4S and ΔDi-6S, an oversulfated disaccharide unit is identified in the chondroitinase digests of chondroitin sulfate E from squid cartilage with an S:N ratio of 1.55. Since the yields of unsaturated disaccharide units are the same in both chondroitinase AC and ABC systems, it may be concluded that this polymer contains no appreciable amounts of L-iduronic acid residues. The susceptibility to chondrosulfatases clearly indicates that O-sulfate groups on this oversulfated disaccharide are located at the positions 4 and 6 of the hexosamine moiety because chondrosulfatases do not cleave the sulfate groups attached to the uronic acid moiety (Fig. 12). Furthermore, even dermatan sulfate with an S:N ratio of 0.94, obtained from bovine lung, contains an oversulfated disaccharide as a minor structural unit, which is a derivative

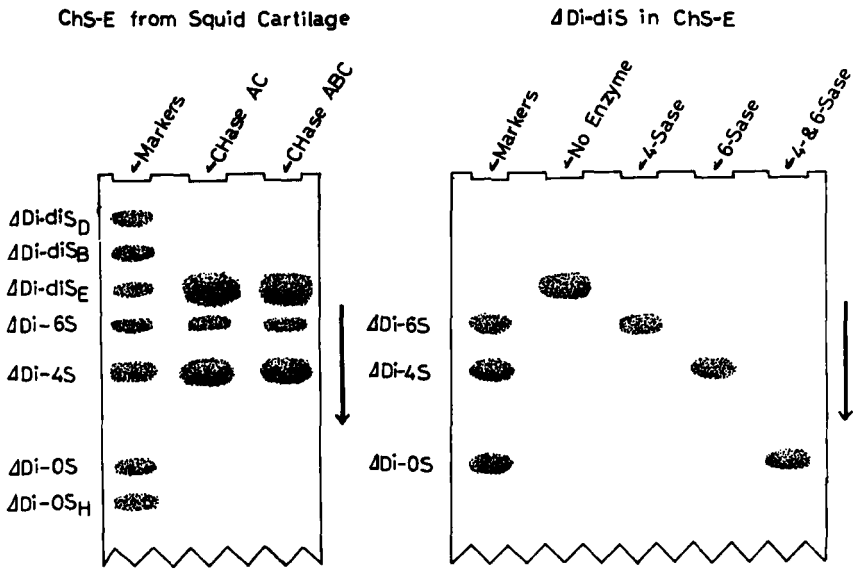

FIGURE 12. Chondroitin sulfate E from squid cartilage is digested with chondroitinase AC and ABC. The disaccharide mapping in n-butyric acid-0.5 M ammonia (5:3, v/v) for 24 h with descending technique shows the same ultraviolet-absorbing spots in both digestion systems (left panel). Thus, this polymer may contain no iduronic acid residues. The spot corresponding in mobility with ΔDi-diS$_E$ is further subjected to the digestion with chondro-4- and chondro-6-sulfatases, yielding ΔDi-6S and ΔDi-4S, respectively. On digestion with chondro-4-sulfatase plus chondro-6-sulfatase, it is converted to ΔDi-0S (right panel). Therefore, it may be concluded that this disaccharide is a derivative of ΔDi-0S bearing two O-sulfate residues at the 4 and 6 positions of the galactosamine moiety. The conditions for enzymatic digestion are the same as in ref. 89 and 90. (from Suzuki et al., ref. 11).

of 4-sulfated disaccharide bearing another sulfate residue on the 2 or 3 position of the iduronic acid moiety, ΔDi-diS$_B$.[11]

With these procedures, the presence of the disulfated disaccharide subunits is also verified in a chondroitin polysulfate of king crab cartilage (ChS-K),[100] a dermatan polysulfate of hagfish notochord (ChS-H),[85] and a dermatan sulfate from human aorta[101] (Fig. 13). In addition, an unique trisulfated disaccharide unit is

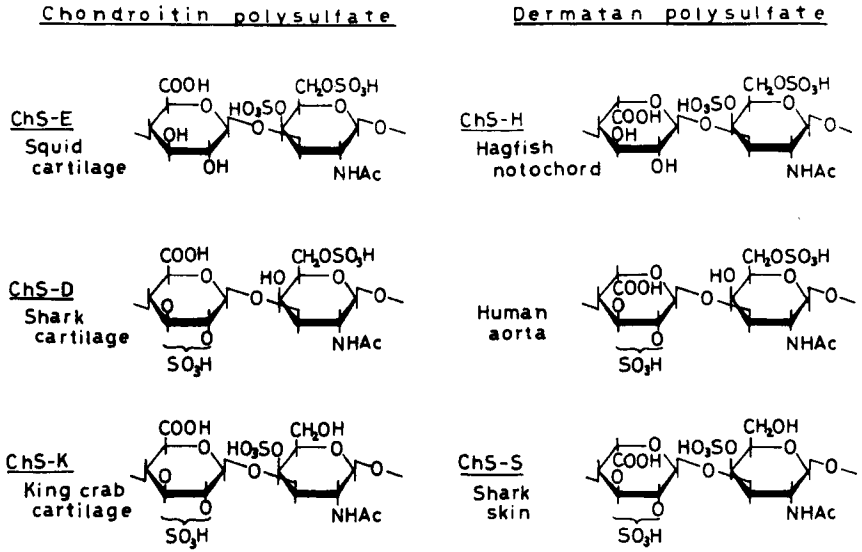

FIGURE 13. Chondroitin polysulfates and dermatan polysulfate containing disulfated disaccharide as one of the major repeating units. (contributed mainly Suzuki et al., ref. 11, and Seno and co-workers, ref. 100, 101, and 85).

demonstrated, for the first time, in a dermatan polysulfate of hagfish skin.[102]

(c) Hybrid structure of chondroitin sulfate isomers

As mentioned previously, the hybrid nature of dermatan sulfate is indicated by the presence of oligosaccharides containing both L-iduronic acid and D-glucuronic acid residues in the chondroitinase digests of various dermatan sulfates.[8,9] However, it would appear that the dermatan sulfate preparations from various sources are still far from homogeneous and the development of more accurate and efficient methods of analysis is necessary for the further structural study of dermatan sulfate.

It has been established that chondroitinase ABC cleaves dermatan sulfate and the chondroitin sulfates at their β-hexosaminidic bonds to either L-iduronic acid or D-glucuronic acid residues to

produce unsaturated disaccharides, while chondroitinase AC cleaves only the bonds to D-glucuronic acid . Degradation of a dermatan sulfate preparation containing some D-glucuronic acid-bearing sections with chondroitinase AC would yield hybrid oligosaccharides with one or more iduronic acid residues and one non-reducing terminal $\Delta^{4,5}$-glucuronic acid, whereas the digestion products with chondroitinase ABC should be composed only of unsaturated disaccharides. When these hybrid oligosaccharides are prepared on a large scale and further subjected to the digestion with chondroitinase ABC or other analyses, the nature of hybrid structure in dermatan sulfate might be confirmed. Thus, various hybrid oligosaccharides are identified in the dermatan sulfate preparations from human articular meniscus,[103] skin and intervertebral disc[12] (Fig. 14).

Though chondroitin sulfates, by definition, contain no iduronic acid residues, another type of hybrid structure is verified in a chondroitin sulfate preparation from bovine tracheal cartilage.[10] This polymer migrates as a single spot between chondroitin 4-sulfate and chondroitin 6-sulfate by electrophoresis in calcium acetate, suggesting a hybrid nature of this polymer. After digestion with testicular hyaluronidase under a condition in which transglycosylation hardly occurs, this polymer yields three types of tetrasaccharides. Desulfation of tetrasaccharide with chondro-4-sulfatase is a useful technique for differentiating sulfated tetrasaccharides.[99] Structural studies on these tetrasaccahrides by the use of chondro-4-sulfatase, followed by chondroitinase, showed that one of them is a hybrid composed of both 4-sulfated and 6-sulfated disaccharides.

(d) Biosynthesis of chondroitin sulfates

Until recently, despite the availability of many analytical procedures, no convincing method has been described for the quantitative and qualitative analyses of extremely small amounts of labeled chondroitin sulfate isomers in the tissue. The disaccharide mapping method of chondroitinase digests appears particularly promising when applied to labeled specimens, since it is possible to determine the relative amounts of the individual chondroitin sulfate

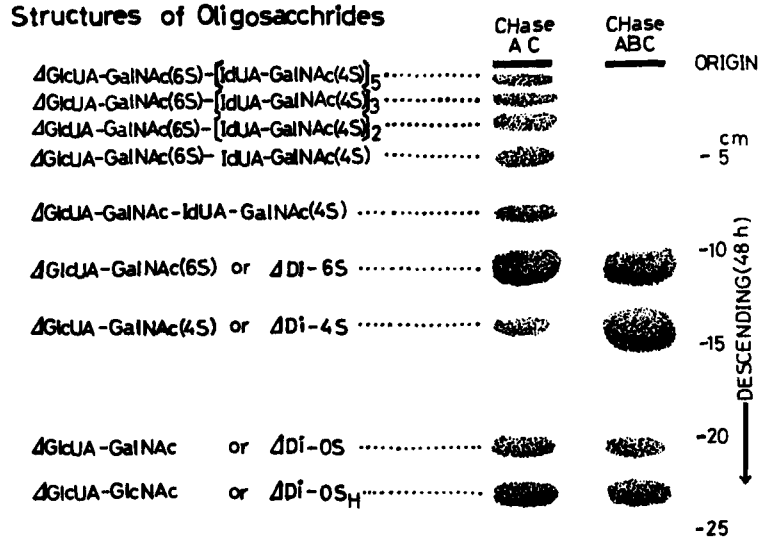

FIGURE 14. Hybrid oligosaccharides in dermatan sulfate from human articular meniscus. Digestion products of crude glycosaminoglycans from joint meniscus with chondroitinase AC and ABC are separated by paper chromatography in n-butyric acid-0.5 M ammonia (5:3, v/v) for 48 h. The conditions for enzymatic digestion and the separation of digestion products are the same as in ref. 89 and 90. Each oligosaccharide in chondroitinase AC-digests is prepared on a large scale by chromatography. The structure of these oligosaccharides is then determined after the digestion with chondroitinase ABC and with Flavobacterium glucuronidase. The abbreviations are: IdUA, L-iduronic acid; ΔGlcUA, $\Delta^{4,5}$-glucuronic acid; GalNAc, N-acetylgalactosamine; GalNAc(4S), N-acetylgalactosamine 4-sulfate; GalNAc(6S), N-acetylgalactosamine 6-sulfate; and GlcNAc, N-acetylglucosamine. (from Habuchi et al., ref. 103).

isomers by measuring radioactivity alone even when there is insufficient material to measure colorimetrically. A mixture of ^{14}C-acetate- or $^{35}SO_4$- labeled chondroitin sulfate isomers is subjected to chondroitinase and the digestion products are applied on filter paper with unlabeled disaccharides as internal markers. After chromatography, the areas corresponding to reference markers are located under ultraviolet light, cut out, and assayed for radio-

activity. The relative amounts of each isomer are calculated from the assay results. By the use of this procedure, Robinson and Dorfman[104] reported that epiphyseal cartilage of 13-day-old chick embryos synthesizes ΔDi-0S, ΔDi-4S, and ΔDi-6S at rates of 20%, 43%, and 37%, respectively. The ΔDi-0S synthesized in this way probably arises from the undersulfated chondroitin sulfates rather than chondroitin itself.

Since the chondroitinase attacks the bond between O and C-4 of uronic acid in an elimination reaction, either saturated disaccharide (if the terminal group is uronic acid) or saturated monosaccharide (if the terminal group is N-acetylgalactosamine or its sulfate) is released from the non-reducing end of substrate after exhaustive digestion. Unsaturated disaccharides are released from the rest of chain. Polysaccharides with different chain length, therefore, vary in their ratios of the saturated products to the unsaturated products. Such ratios give a measure of the degree of polymerization of the polymer. This method thus may be applied to the study of chain elongation mechanism of chondroitin sulfate with the use of labeled compounds.[105]

Heparinase and heparitinase

The structure of heparin and heparan sulfate is more complex than those of the other glycosaminoglycans and the exact arrangement of the subunits is not known in details. These difficulties in deciding the structures of these polymers have been due mainly to the well-known fact that these polysaccharides are resistant to acid hydrolysis and the structural determination of polysaccharide has almost invariably involved acid hydrolysis to yield oligosaccharides which are amenable to structural analysis.

Recently, a heparinase and two types of heparitinase are isolated from extracts of induced Flavobacterium heparinum.[94] As the conditions of enzymatic digestion are mild compared to those of acid hydrolysis, it is thus possible to isolate sulfated oligosaccharides in good yield. The heparinase acts directly upon

heparin, yielding 52% of a trisulfated disaccharide and 40% of a tetrasaccharide beside small amounts of hexasaccharide. The tetrasaccharide is in turn degraded by heparitinase II, forming nearly equal amounts of trisulfated and disulfated disaccharides. These results indicate that the tri- and disulfated disaccharides are linked alternatively in a proportion of 3:1, respectively. The trisulfated disaccharide is the major subunit of heparin comprising about 70-80% of the total structure and the uronic acid residue of this disaccharide is L-iduronic. On the other hand, the disulfated disaccharide is about 20% of the total and the uronic acid seems to be D-glucuronic. Tentative structure of heparin and the mode of action of heparinase and heparitinase II are proposed based on the analysis of the different digestion products with these enzymes[106] (Fig. 15). Silva and Dietrich[94] suggested that heparinase and heparitinase II may be specific for N-sulfated glucosamine α-1,4 iduronosyl and N-sulfated glucosamine α-1,4 glucuronosyl linkages, respectively. On the other hand, heparitinase I may cleave N-acetylglucosamine α-1,4 glucuronosyl bond.

It has been thought that the hexosamine and the uronic acid alternate in heparin and both glucosidic linkages are α-1,4. However, though not conclusive, new evidence is presented that a large portion of the glucuronidic linkages of heparin and heparan sulfate may have β-configuration and the L-iduronidic linkages may have α-configuration.[4,6]

Keratan sulfate-endogalactosidase

The presence of keratan sulfate-degrading enzymes in bacteria is reported by several workers.[107,108] Recently, Nakazawa and Suzuki[109] reported that a glycosidase which attacks corneal keratan sulfate is isolated from extracts of adapted Pseudomonas sp. IFO-13309. This enzyme produces scissions at galactosidic bonds along the corneal keratan sulfate chain and hence can be classified as an endo-β-galactosidase. This enzyme attacks only the endo-β-galactoside bonds in which non-sulfated D-galactose residues participate.

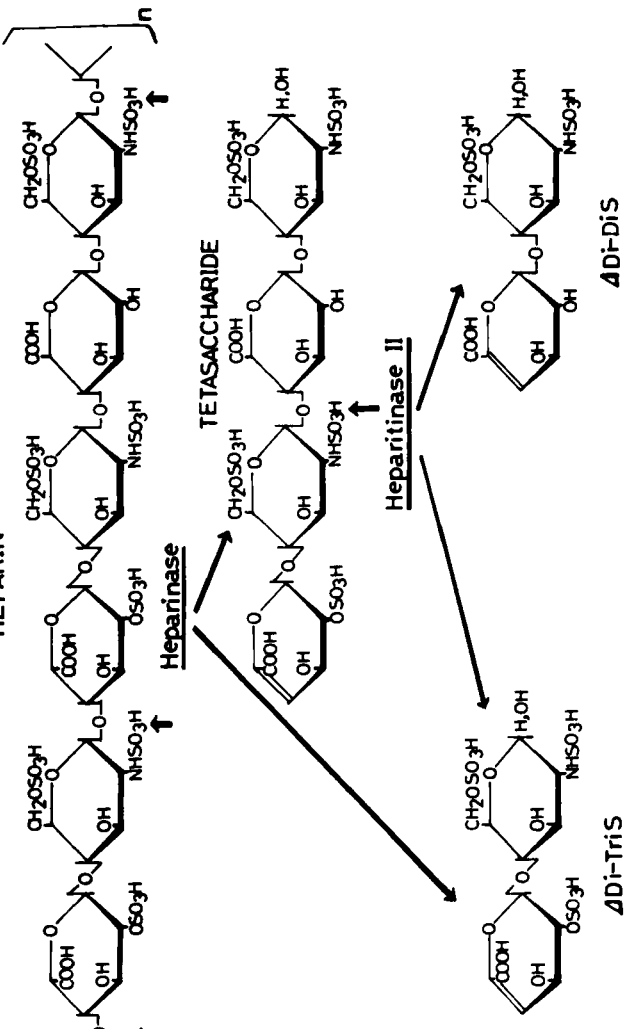

FIGURE 15. Proposed structure and mode of action of heparinase and heparitinase II in the digestion of heparin to unsaturated disaccharides. ΔDi-TriS and ΔDi-DiS; unsaturated tri- and disulfated disaccharides. The nature of uronidic linkages has not yet been determined with certainty. (from Silva and Dietrich, ref. 106.)

Substitution of the galactose moiety at the 6 position with a sulfate ester group impairs hydrolysis. It has been demonstrated that about 40% of the galactosyl groups in corneal keratan sulfate are substituted at the 6 position.[110] If these sulfated galactosyl groups are distributed unevenly along the polysaccharide chain, treatment with this enzyme would result in a formation of fragments with varying chain length, some of which are subsequently attacked by a specific sulfatase isolated from adapted Actinobacillus extracts.[111]

Compared to corneal keratan sulfate, cartilage keratan sulfates are attacked at lower rates with a resultant production of oligosaccharides of relatively large size. This result is agreement with the view that considerable variations exist in the structure of keratan sulfates of different origin. This enzyme may serve as a useful tool in studying these variations.

Great strides in the chemistry of glycosaminoglycans have been made by the application of enzymatic methods. Commercially available preparations of substrate-specific enzymes, such as chondroitinases, chondrosulfatases, and Streptomyces hyaluronidase, provide the sensitive and accurate methods for the identification and the structural analysis of various polysaccharides, especially chondroitin sulfate isomers. It should be emphasized that the application of these enzymatic methods is to be regarded as an indispensable part of the analytical process without which a full spectrum of information cannot be obtained.

The purified preparations of chondroitinase AC and ABC, chondro-4-sulfatase, chondro-6-sulfatase, and Streptomyces hyaluronidase (AMANO) are all now available from Seikagaku Kogyo Co. LTD., 2-9, Nihonbashi-Honcho, Chuo-Ku, Tokyo, Japan, or from Reseach Product Division of Miles Laboratories Inc., Elkhart, Ind. 46514, U.S.A.

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