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REVIEW

EXTRACELLULAR MAMMALIAN POLYSACCHARIDES: GLYCOSAMINOGLYCANS AND PROTEOGLYCANS

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1. INTRODUCTION

The extracellular matrix is composed primarily of collagen, elastin and proteoglycans. It is of interest because of its ubiquity within mammals and because its malfunction is involved in common chronic disease processes for which there is no reliable cure. The principal polysaccharides of the extracellular matrix are termed proteoglycans. They consist of a protein core with numerous covalently linked glycosaminoglycan (GAG) side-chains. Naturally occurring mammalian GAGs and the slightly older term acid mucopolysaccharide are synonymous in mammalian systems, each referring to one of five principal polymers; hyaluronate, chondroitin, keratan, dermatan or heparan. Recent reviews of these molecules [1–3] cover the structure, synthesis and degradation in appreciable detail.

The separation of GAGs from mammalian tissues and fluids has a lengthy history [4]. A landmark event, however, was the demonstration by J.E. Scott [5,6] of polysaccharide precipitation by quaternary ammonium compounds, thus preparing the way for the field of proteoglycan research. This review is intended to cover recent advances in the biochemistry of the GAGs and proteoglycans, with emphasis on separation techniques and biomedical applications.

The methods which will be reviewed here are electrophoresis (by agarose, acrylamide and cellulose acetate), high-performance liquid chromatography (HPLC); for molecular sieving and disaccharide analysis) and conventional column chromatography (ion exchange, gel permeation and affinity). Since detection systems are an integral part of modern separation technology, the best methods for assaying GAGs or detecting column fractions will also be discussed.

The medical relevance of these procedures becomes obvious in the section on associated disease states. For example separation and identification of GAGs are prerequisites for confirming the diagnosis of mucopolysaccharidoses. In addition, the identification of individual GAGs is also proving to be valuable in arthritis research and in studies of other connective tissue diseases.

The selection of the citations to be reviewed here was restricted to those articles describing new GAG separation techniques or analytical methods which might

lead to new technical developments. Specifically omitted from this review were medically related reports which were not concerned with GAG separation technology. Also omitted were applied clinical research reports whose primary concern appeared to be the promotion of commercial products.

2. STRUCTURE

The basis of separation and the design of separation methodology entails a thorough understanding of the chemical structure of the molecule. Accordingly, each of the five structural GAG classes is summarized.

2.1. Hyaluronate

Hyaluronate is composed of alternating β-1–3 glucuronidic and β-1–4 N-acetylglucosaminidic bonds, repeated ad infinitum.

-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→4)-

where Glc*p*A is glucuronic acid pyranose and Glc*p*NAc is N-acetylglucosamine pyranose.

The primary occurrences in mammalian tissues are in skin, synovial fluid, vitreous fluid and umbilical cord. Non-mammalian sources include cocks comb and streptococcal bacteria. Naturally occurring hyaluronate is believed to be the only GAG not covalently attached to protein.

The single outstanding feature of hyaluronate is its ability to form a stable aqueous gel at chemically low concentrations. Physiologically, however, hyaluronate gels are only known to exist in the presence of collagen. When collagen is absent or in low concentration as in the vitreous of the owl monkey eye, no gel is formed [7].

Sodium hyaluronate has a very enhanced circular dichroism (CD) spectrum relative to its constituent monosaccharides. This can be attributed to the β-1–4 linkage from N-acetyl-D-glucosamine to D-glucuronate. The principal CD band is negative at 209 nm and its intensity is dependent on the chain length. As the chain length decreases, the molar elipticity becomes less negative. Polymeric hyaluronate molar elipticity values are $-10\,200 \pm 500$ while oligosaccharides are approximately -7500 ± 300 deg cm^2 dmol^{-1} [8].

The gel state of a hyaluronate solution is clearly affected by the solution pH. At neutral pH a low-percent solution of high-molecular-mass material might display a viscosity between 500 and 1000 cP. As the pH drops to between 3 and 4 a transition state is achieved yielding a gel state, which reverts to a thin liquid at yet lower pH values. Above pH 7, the viscosity progressively decreases. At pH 12.5 a sharp, but reversible, drop in viscosity occurs [9]. The decrease in radius of gyration and concomitant conformational changes are believed to be specific to hyaluronate. Both light scattering and CD studies, used to investigate these changes, suggest a left-handed chirality for the solubilized polymer and protonation of the glucuronate carboxyl as the explanation for the multiple phenomena [9–11]. Currently, the hyaluronate molecule is considered to be a double helix

held together by extensive hydrogen bonding and hydration bridges [12]. Indeed the hydration sphere of the dimeric unit, both within the intact hyaluronate and as a separate entity, has been studied by compressibility and density measurements [13]. The results indicate that the glucuronate residue is significantly more hydrated in the polymer than is the N-acetylglucosamine, and that the polymer is less hydrated than the individual monosaccharides. Counterions, particularly sodium, potassium and calcium, also effect the overall hydration and conformation of the molecule. X-Ray fiber diffraction clearly shows the changes occurring with pH, the state of hydration and the change in counterion. The neutral sodium hyaluronate unit cell approximates a four-fold helix with no requirement for water molecules [14]. Upon introduction of potassium, the axial rise per disaccharide increases from 0.84 to 0.89 nm [14–16] and a requirement of four water molecules per disaccharide is imposed. The introduction of substoichiometric amounts of calcium cause the formation of the calcium complex, which switches the molecule to a three-fold helix with a 0.95-nm axial rise per disaccharide. Further, the unit cell now requires nine water molecules per disaccharide to support the crystalline state [14].

2.2. Chondroitin sulfate

Chondroitin sulfate consists of alternating β-1–3 glucuronidic and β-1–4 N-acetylgalactosaminidic bonds, and is sulfated at either C-4 or C-6 of the N-acetylgalactosamine pyranose (Gal*p*NAc). It is located in mammalian tissues in skin, bone, cornea and especially cartilage.

$$-(1\rightarrow4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow3)\text{-}\beta\text{-D-Gal}p\text{NAc}(4\text{-}SO_3^-)\text{-}(1\rightarrow4)\text{-}$$

$$-(1\rightarrow4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow3)\text{-}\beta\text{-D-Gal}p\text{NAc}(6\text{-}SO_3^-)\text{-}(1\rightarrow4)\text{-}$$

In vivo, chondroitin sulfate attaches covalently to protein through a xylose–serine linkage in which the linkage region contains the tetrasaccharide glucuronosyl–galactosyl–galactosyl–xylose [17].

Due to a much lower molecular mass of naturally occurring chondroitin sulfate, 10 000–50 000 [1], the viscosity behavior of chondroitin sulfate is apparently Newtonian. Concentrations of 20–50% do not exhibit high viscosities. This differs markedly from the behavior of hyaluronate. For example, in one study a 50% chondroitin sulfate preparation had an absolute viscosity of 900 cP while a 1% sodium hyaluronate preparation had a viscosity of 32 000 cP [18].

X-Ray analysis of purified chondroitin 4-sulfate (chondroitin sulfate A) has been performed. The sodium salt has a three-fold helical structure which in the presence of small amounts of divalent calcium converts to a two-fold helix. The potassium salt can adopt either the three-fold or two-fold conformation [19,20].

2.3. Dermatan sulfate

Dermatan sulfate consists of alternating β-1–3 glucuronidic (or α-L iduronidic) and β-1–4 N-acetylgalactosaminidic bonds, and is sulfated at either C-4 or

C-6 of the N-acetylgalactosamine. The uronic acid residue is variably either iduronic or glucuronic acid with the percentage of each dependent on the source. Further, the iduronic acid may be sulfated or not in the C-2 position.

-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc(4-SO_3^-)-(1→4)-

-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc(6-SO_3^-)-(1→4)-

-(1→4)-α-L-Ido*p*A-(1→3)-β-D-Gal*p*NAc(4-SO_3^-)-(1→4)-

-(1→4)-α-L-Ido*p*A-(1→3)-β-D-Gal*p*NAc(6-SO_3^-)-(1→4)-

-(1→4)-α-L-Ido*p*A(2-SO_3^-)-(1→3)-β-D-Gal*p*NAc(4-SO_3^-)-(1→4)-

-(1→4)-α-L-Ido*p*A(2-SO_3^-)-(1→3)-β-D-Gal*p*NAc(6-SO_3^-)-(1→4)-

where Ido*p*A is iduronic acid pyranose.

Analogous to the studies with hyaluronate and chondroitin, X-ray diffraction analysis has provided a refined concept of the molecular conformation. Of three allomorphs observed, one was found to be unique among the GAGs in having right-handed rather than left-handed helices [21]. Some minor controversy exists yet over the chair-boat conformation of the iduronate moiety [22,23]. There is evidence that the actual conformation is predominantly a mixture of chair forms as well as one boat form [24].

2.4. Heparan sulfate

Heparan sulfate consists of alternating β-1–4 uronidic and α-1–4 N-acetylglucosaminidic bonds, and is sulfated at either N or C-6 of the N-acetylglucosamine. It is the only GAG with invariable 1→4 linkages rather than alternating 1→3 and 1→4 linkages between sugars. It is also the only GAG with an α-D linkage position between the N-acetyl sugar and the uronic acid. As with dermatan sulfate, the uronic acid residue is a mixture of either iduronic or glucuronic acid, dependent upon the source of material, with the iduronic acid variably sulfated in the C-2 position.

-(1→4)-β-D-Glc*p*A-(1→4)-α-D-Glc*p*NAc-(1→4)-

-(1→4)-β-D-Glc*p*A-(1→4)-α-D-Glc*p*NSO_3^--(1→4)-

-(1→4)-α-L-Ido*p*A-(1→4)-α-D-Glc*p*NAc-(1→4)-

-(1→4)-α-L-Ido*p*A-(1→4)-α-D-Glc*p*NSO_3^--(1→4)-

-(1→4)-α-L-Ido*p*A(2-SO_3^-)-(1→4)-α-D-Glc*p*NAc-(1→4)-

-(1→4)-α-L-Ido*p*A(2-SO_3^-)-(1→4)-α-D-Glc*p*NSO_3^--(1→4)-

Heparin is distinguished from heparan sulfate in that heparin is the trisulfate possessing SO_3^- at C-2 of the uronic acid, and at the N and C-6 of the glucosamine residue. Heparin is also the only sulfated GAG existing free in the extracellular matrix and not specifically associated with protein or within a proteoglycan. Both species are principally found in lung and liver tissue, with a molecular mass range of 10 000–80 000.

2.5. Keratan sulfate

Keratan sulfate consists of alternating β-1–4 galactosyl- and β-1–3 N-acetylglucosamine residues. The dimer is also known as N-acetyllactosamine. It may be sulfated or not at the C-6 position of either sugar.

-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-

-(1→3)-β-D-Gal*p*(6-SO_3^-)-(1→4)-β-D-Glc*p*NAc-(1→3)-

-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc(6-SO_3^-)-(1→3)-

-(1→3)-β-D-Gal*p*(6-SO_3^-)-(1→4)-β-D-Glc*p*NAc(6-SO_3^-)-(1→3)-

Keratan sulfate is linked to protein through either a β-N-acetylglucosamine–asparagine linkage (keratan sulfate I) or a Gal(1,6)-α-N-acetylgalactosamine–threonine (or serine) as in keratan sulfate II. Type I is located only in the cornea, while skeletal tissue possesses both [17]. Keratans are unique among the GAGs in that uronic acid is absent, and the molecular mass is quite low, 2000–20 000.

2.6. Proteoglycans

A proteoglycan in simplest form consists of protein covalently connected to GAG. All other polysaccharide protein entities are classified as glycoproteins. In general, proteoglycans have a greater glycan content and a lesser protein content while for glycoproteins this concept is reversed. Three types of carbohydrate protein linkages are found. An O-glycosidic linkage can exist between N-acetylgalactosamine and the hydroxyls of threonine or serine. The amide of asparagine can form a C-1–N bond to N-acetylglucosamine. These two linkages are unique to keratan sulfate II and I, respectively. The third type which is found for chondroitin, dermatan and heparan is an O-glycosidic bond between D-xylose and a serine hydroxyl group.

Electron microscopy has yielded a bottle brush structure for aggregates of proteoglycans [17,25]. This structure is routinely interpreted as a hyaluronate core, lined with non-covalently bound but tightly associated link proteins, which in turn attach non-covalently to proteoglycans. The principal tissue source of proteoglycan is cartilage, although much of the scientific work has been carried out in swarm rat chondrosarcoma, an easily propagated cartilage-derived tumor. The tumor is noted by the absence of keratan sulfate.

Why does the proteoglycan monomeric structure fail to include hyaluronate? Although the definitive experiment has not been done, hyaluronate does not covalently attach to protein. Evidence for a covalent protein–hyaluronate linkage may be explained by incomplete hyaluronate purification techniques designed to preserve putative O-glycosidic protein linkages. Alternatively, since the synthesis of the exceptionally large hyaluronate molecule is not template directed as is DNA, one might expect residue errors, some of which might permit protein linkages. It can be shown that the biosynthesis of hyaluronate is not inhibited by protein synthesis inhibitors such as puromycin or cycloheximide [26], and that monensin, which perturbs ionic transport over the Golgi membranes, inhibits the biosynthesis of chondroitin sulfate but not that of hyaluronate [27]. This evidence suggests that hyaluronate is not synthesized by the same intracellular route as are glycoproteins and proteoglycans. Indeed, the solubilization and resultant purification of hyaluronic acid synthetic activity has been achieved only recently [28]. Future mechanistic studies of hyaluronate synthetic activity should elucidate the enzymatic roles, the error rate for residue placement and reasons for the absence of hyaluronate in proteoglycans.

3. DETECTION METHODS

The key to a successful biochemical separation is a specific assay. As such, what follows is first a review of detection systems and assays. There are four principal methods of detecting GAGs. The first group includes Alcian blue and Methylene blue dye binding, bovine serum albumin (BSA) polysaccharide affinity, monoclonal antibody systems and a wide variety of radioactive methods which preserve the intact polymer. The second group of methods detect the non-polymerized carbohydrate components of GAGs. Included in this group are stains such as *p*-dimethylaminobenzaldehyde, Toluidine blue, carbazole and indole. The third detection method is ultraviolet (UV) spectrophotometry and the fourth is refractometry. In continuous monitoring of column chromatography, the latter two methods are especially useful.

3.1. Polysaccharide analysis

Alcian blue dye (Alcian blue 8Gx, a copper phthalocyanin dye) [29] is the most commonly used reagent for detection of GAGs. The blue dye–GAG complex is insoluble at netural pH. Thus the basis of detection is the specific removal of GAG from solution. As is the case with many dyes, reaction conditions are extremely critical. Some of the factors which are known to make a significant difference include ionic strength, Mg^{2+} concentration, temperature, time at temperature, dye concentration, solvent concentration, pH, aggregation state of dye in solution and presence of biological competitive inhibitors. Undoubtedly the list could be extended. Alcian blue was popularized as a selective stain for mucin by Scott [6,30], who also suggested that it was suitable for use in his critical electrolyte concentration method for quantitating polyanions. However, one must

carefully control all of the items listed above before attempting to quantitate this method [31,32].

A very sensitive, albeit slow, method for the quantitative estimation of GAG involves staining with Alcian blue and using atomic absorption to monitor for copper [33]. The method is particularly suitable after cellulose acetate electrophoresis of biological fluid samples where contaminating copper complexes could be presumed absent. The sensitivity is of the order of 15–190 ng of uronic acid. It is notable that if the electrophoretic band is not visible, accurate quantitation is unlikely.

An extremely simple and unusually direct method for the Alcian quantitation of soluble GAG has been proposed by Gold [34]. Using 0.5 *M* sodium acetate as solvent, the Alcian dye and dye–GAG complex remain soluble. Increases in the absorption spectrum of the dye complex at 480 nm provide a convenient detection of GAG. Sample (0.1 ml containing 5–75 μg of GAG) is combined with 1.2 ml of dye solution (1.4 mg/ml in 0.5 *M* sodium acetate, made fresh and not filtered). After 10 min the 480-nm absorbance is read and compared to standards. Absorption is linear with concentration and stable between 10 and 30 min but varies in intensity among purified GAGs. A single objection to this method is a low absorption difference (0–0.35), which results in lowered precision. Still, it is an excellent, quick assay for the estimation of total GAG in solution. By performing essentially the same method but with 15% concentrated phosphoric acid, 2% concentrated sulfuric acid and 1 mg/ml Alcian blue dye as the dye solution, a similar estimate of only the sulfated GAGs is achieved [35]. A slightly more complex, but more sensitive, Alcian method is described by Bartold and Page [36]. Their method involves spotting the sample onto cellulose acetate, permitting detection of GAG after electrophoresis. The spot is stained, removed, solubilized with dimethyl sulfoxide, and quantitated at 678 nm in a spectrophotometer. The sensitivity limit is about 1 μg of GAG.

Since neutral Alcian blue solutions bind, aggregate and remove polysaccharide from solution, monitoring the decrease of solubilized dye provides a simple and more precise assay for total GAG. A 1-ml volume of an Alcian blue dye reagent [0.45 *M* sodium acetate, 50 m*M* magnesium chloride, 0.1% (w/v) Alcian blue dye] may be added to 250 μl of sample containing 5–80 μg of GAG. This mixture is mixed gently for 10 min, then spun in a microcentrifuge (3000 *g* for 3 min) to pellet the GAG–Alcian aggregate. To 0.5 ml of the supernatant, 3.0 ml of 0.9% sodium chloride are added, mixed, and the absorbance at 620 nm is determined. Quantitation is achieved using a standard curve. The assay is based on an absorbance decrease and the blank must be set arbitrarily at about 1 A.U. However, accuracy and precision are greatly enhanced by decreased sample handling and the use of the full 0–1 absorption scale [37,38]. Recently it has been shown that very short oligosaccharides, containing seven disaccharide units or less, do not bind the dye. Depending on the solvent conditions, dye binding is linear between 12 and 30 disaccharide units [39].

An infrequently used dye, capable of measuring GAG content, is 1,9-dimethylmethylene blue [40,41]. While it has been suggested that intense stain differences for this dye exist among the GAGs, such a property could lead to a selective

advantage when working with impure materials [42]. Another advantage of 1,9-dimethylmethylene blue is that it is obtained chemically pure as opposed to most dyes which are at best 90% pure. Thus, staining might lead to more precise quantitation.

A particularly sensitive detection system for GAG spots on cellulose acetate and agarose gels utilizes BSA and Coomassie brilliant blue R-250 [43]. BSA has an affinity for GAG which greatly exceeds its affinity for the cellulosic electrophoretic media. After running the electrophoresis in any of a variety of systems, the color is achieved by binding BSA to the GAG, and then washing away the unbound albumin and staining the GAG–albumin complex with Coomassie blue. The limit of detection is 15–40 ng on cellulose acetate and 50–150 ng in agarose. Clearly in these media, this method is superior to the use of Alcian blue or Toluidine blue, since both of the latter dyes produce an intense background stain which limits their sensitivity.

The most sensitive detection scheme is to use a radioactive tracer or tag. Obviously, one could inject radiolabeled GAG precursors in vivo and thereby obtain labeled GAGs [44–46]. However, with the exception of cell or organ culture systems [47,48], most laboratories consider that labeling technique extremely inconvenient. In some instances, it is very helpful to synthetically tag GAG. This has been accomplished for hyaluronate through the use of hydrazine to partially deacetylate the polymer followed by reacetylation with tritiated acetic anhydride [49]. A potential objection to this method is that the labeling conditions reduce the hyaluronate chain length. As such, it may no longer behave within an in vivo pool as other more highly polymerized hyaluronate molecules. Still it is an exceptionally useful tool for any application in which chain length is not considered critical. The acetylation technique is also very adaptable to other purified GAGs.

Two autoradiographic methods for use with cellulose acetate strips have appeared. The first uses ^{103}Ruthenium red dye as a GAG stain and exhibits a detection level of 2–4 ng per spot [50]. A disadvantage is that the Ruthenium dye does not adhere well to hyaluronate or keratan sulfate. The second and yet more sensitive method (1 ng per spot) utilizes the basic protein [^{125}I]cytochrome C, which has a high affinity for GAGs [51]. This latter method appears sensitive to all five GAGs although hyaluronate, which lacks a low-pK sulfate group, is not as well detected.

A much used radioactive assay for hyaluronate is that of Laurent and Tengblad [52]. This competitive binding assay measures hyaluronate by its inhibition of the binding of ^{125}I-link protein to a hyaluronate-Sepharose gel. The method is tedious but specific, even in the presence of gross impurities. Its major disadvantage is that the link protein is not currently commercially available and must be purified and radiolabeled in order to perform the assay.

The use of monoclonal antibodies as specific probes of proteoglycans and GAGs is just emerging [53,54]. The interest in these is strong because of well founded speculation that common pathological conditions result in changes to the extracellular matrix proteins and polysaccharides. These methods have thus far been used primarily in histological studies, but their potential for analytical application is substantial. Due to the extraordinary specificity of the antibodies, the

sensitivity of the technique is superior for the quantitative detection of proteoglycans.

3.2. Carbohydrate analysis

The carbazole reaction was developed in 1947 by Dische [55] for analyzing hexuronic acid. The method most frequently followed today uses the modification of Bitter and Muir [56]. The method relies on acid hydrolysis of GAG to produce the component sugars followed by derivatization with carbazole and colorimetric detection. Since iduronic acid and glucuronic acid do not develop the same amount of color, an appropriate standard should be used dependent upon the GAG assayed. Precision of the assay is difficult to achieve because the derivatization results in more than one chromophore, requiring tightly controlled reaction conditions to obtain a consistent chromophore production. Also, the acid hydrolysis must be quantitative, without degradation of the uronic acid. Enhanced precision has been reported using an automated method [57], which has also been modified to use the dye reagent *m*-hydroxydiphenyl [58]. This automated procedure is suitable as a continuous-flow monitor of gel permeation columns [59].

A parallel approach for the detection of GAGs is to assay for hexosamine, using the method of Elson and Morgan [60]. As with the carbazole reaction, an automated method has improved the precision [61]. The basis of the reaction, after complete polymer hydrolysis, is an initial condensation between the amine portion of the hexosamine and acetylacetone. This is followed by a second condensation with *p*-dimethylaminobenzaldehyde to produce the color.

An alternative colorimetric assay for amino sugars uses indole hydrochloride [62]. A modification of the original method has recently appeared [63] and is very easy. The assay involves a 15-min acid hydrolysis of the GAG to deacetylate the hexosamine, treatment at room temperature with sodium nitrate to cleave residual glycosaminidic linkages and treatment with indole at 100 °C for 5 min to obtain the chromophore. Measurement of the absorbance at 492 nm monitors the extent of hydrolysis and formation of chromophore. A suitable standard for the GAGs is N-acetylglucosamine or N-acetylgalactosamine. The method is sensitive (15–75 nmol of hexosamine per assay tube) and can be easily used to monitor column fractions.

3.3. Instrumentation

Specific assays can be developed for each of the GAGs based on the specificity of enzymic lyases. For example, *Streptomyces hyalurolyticus* produces an eliminase (hyaluronate lyase) which specifically hydrolyzes hyaluronate to oligosaccharides. Each oligosaccharide contains one terminal Δ-4,5-unsaturated glucopyranuronic acid absorbing at 232 nm. For neat systems this is an adequate assay since the enzyme contributes negligibly to the absorbance. Unfortunately, most uses require detection in the presence of other strongly UV-absorbing compounds. However, the Δ-4,5-unsaturated uronic acid will react with periodate sulfuric acid followed by thiobarbituric acid to yield a chromophore absorbing at 549

nm [64,65]. Most biological tissue or fluid systems have very low endogenous 549-nm absorbance, thus permitting the use of this assay. A well characterized hyaluronate standard is required, since several oligosaccharide chain lengths are produced and the stoichiometry, even with complete digestion, is not well controlled. A similar method exists for chondroitin sulfate and dermatan sulfate [66].

Obviously the above methods do not offer the quintessent solution to the problem of detecting fractions containing GAGs or proteoglycans. An alternative method which can be used in many instances is differential refractometry. Many commercial instruments are available. The differential signal produced is the difference between the refractive indices of the sample and the solvent and is proportional to concentration. The lower limit of detection has been reduced by modern instrumentation and is now in the range of 0.01 mg/ml. A significant disadvantage, of course, is that refractometry is completely non-specific, and anything that differs from the refractive index of the solvent is detected.

A new instrument, which has recently been commercialized, is a differential viscometer. It measures the difference between solvent viscosity and sample viscosity in a manner analogous to the differential refractometer, except that the solutions are put through a capillary tube version of a Wheatstone bridge with a pressure transducer as the bridge element. The interest in this instrument is that the output is directly proportional to specific viscosity. Specific viscosity data can be transformed into more informative intrinsic viscosity and molecular-weight data via the Mark–Houwink equation $[n] = KM^a$, where K and a are predetermined constants, and $[n]$ and M are intrinsic viscosity and molecular weight, respectively. The advantage is obvious in size-exclusion chromatography, since a single chromatograph in which both a differential refractometer and a differential viscometer are used will yield a molecular weight of the polymer. The detection limit of this system is seemingly dependent upon both concentration and viscosity, but typical useful concentrations are greater than 0.05 mg/ml [67, 68].

4. SEPARATION METHODS

Recent advances are discussed for ion-exchange, gel permeation and hydrophobic interaction chromatography, HPLC, electrophoresis, gas chromatography and biospecific affinity chromatography. Enzymatic methods including separation through the use of proteases, nucleases and especially GAG lyases are covered in a separate section.

Detailed analytical methods are not presented here. Rather the discussion is intended as a critique and compilation of existing GAG methodology. For specific instruction in performance of these procedures, the citations must be referenced.

4.1. Separation strategy for proteoglycans and glycosaminoglycans

Proteoglycans are normally extracted from cartilage tissue using chaotropic solvent conditions such as 8 *M* urea, 4 *M* quanidine, or 3 *M* magnesium chloride. Such conditions promote dissociation of charge interactions, destabilize high-molecular-mass aggregate forms and permit extraction of the majority of the pro-

teoglycans. To separate the proteoglycans from other components of the extract, cesium chloride density gradient centrifugation is commonly used. Ion-exchange chromatography may then be used to further fractionate individual proteoglycans.

Extraction of tissues for the purpose of purifying GAGs is frequently performed in the presence of broad-spectrum proteases such as papain, clostripain or protease K (Table 1). GAGs in the initial extract (except low-molecular-mass keratan sulfate) may be precipitated with a quaternary ammonium salt such as cetylpyridinium chloride (1% in low ionic strength, <0.15 *M* sodium chloride). The remaining keratan sulfate can be isolated from the supernatant by diethylaminoethyl (DEAE) chromatography, from which it is eluted at a relatively high salt concentration (0.65–1 *M* sodium chloride). The cetylpyridinium precipitate, which is greatly enriched in hyaluronate, chondroitin sulfate, dermatan sulfate and heparan sulfate, can be redissolved in 1.0 *M* sodium chloride and reprecipitated with ethanol to reduce the cetylpyridinium salt. After redissolving the ethanol precipitate in water, the GAG fraction can be bound to DEAE-Sephadex and gradient eluted to obtain enriched fractions of individual GAGs. Alternatively, the dissolved ethanol precipitate may be treated with enzymes (see Table 1) to selectively degrade specific GAGs, proteins or nucleic acids prior to a second ethanol precipitation and ion-exchange purification. Since most source materials do not actually contain all five GAGs, these procedures can yield a nearly pure fraction of any chosen GAG.

4.2. Enzymic fragmentation

In the laboratory analysis of GAGs numerous enzymes are used. Proteases, especially papain and protease K, are used prior to GAG extraction to degrade protein. Nucleases, especially DNAase I, effectively degrade nucleic acid during isolation of high-molecular-mass hyaluronate. Analytically, chondroitinases and hyaluronidases are used to identify specific GAG fractions. The most specific degradative enzyme used is hyaluronate lyase from *S. hyalurolyticus* or *S. equisimilis*, a fungal eliminase-type hyaluronidase which produces Δ-4,5-unsaturated tetra- and hexasaccharides. Elimination of a chromatographic peak by this enzyme identifies hyaluronate [69,70]. Testicular hyaluronidase, an endo-β-N-acetylglucosaminidase, degrades hyaluronate, chondroitin sulfates and dermatan sulfate. Limited use of the enzyme will produce a broad spectrum of oligosaccharides, which always contain multiples of Glc*p*A-Glc*p*NAc [8]. Leech head hyaluronidase is an endo-β-glucuronidase (hyaluronate β-glycanohydrolase) whose action produces a Glc*p*NAc–Glc*p*A disaccharide [8]. Of the two common chondroitinases (ABC and AC), chondroitinase ABC is similar in specificity to testicular hyaluronidase. It degrades chondroitin 4- and 6-sulfate and dermatan sulfate more rapidly than hyaluronate, but it does not degrade keratan sulfate, heparan sulfate or heparin [71]. Chondroitinase AC is slightly more specific in that it will not degrade dermatan sulfate. Thus one may confirm the identity of a proteodermatan sulfate fraction by digesting it with chondroitinase ABC and failing to do so with chondroitinase AC [72].

There are also two chondrosulfatases specific for the 4- and 6-sulfate positions

TABLE 1

ENZYMES USED IN GLYCOSAMINOGLYCAN SEPARATION AND ANALYSIS

Group I: Enzymes that are specific to individual classes of glycosaminoglycans; group II: enzymes that have as their substrates entities which are contained in multiple glycosaminoglycan classes; group III: enzymes used as sample pretreatments prior to separation or analysis (except for lipoprotein lipase which is an affinity ligand for heparan sulfate). EC No.: Enzyme Commission Number.

Group	Common enzyme name	Source	EC No.	Action/use	Reference
I	Streptomyces hyaluronidase	*Streptomyces hyalurolyticus*	4.2.2.1	Eliminase-type enzyme; specifically degrades hyaluronic acid	69
	Hyaluronate lyase	*Streptococcus equisimilis*	4.2.2.1	Eliminase-type enzyme; specifically degrades hyaluronic acid	70
	Keratanase	*Pseudomonas*	3.2.1.103	Endo-β-galactosidase; glycanohydrolase; specifically degrades keratan sulfate	77
	Heparitinase	*Flavobacterium heparinum*	4.2.2.8	Specifically degrades heparan sulfates (heparitin A, heparitin B)	80
	α-L-Iduronidase, form A	Human fibroblasts	–	Specifically degrades heparan sulfate	79
	α-L-Irudonidase, form B	Human fibroblasts	–	Specifically degrades dermatan sulfate	79
	Chondro-4-sulfatase	*Proteus vulgaris*	3.1.6.9	Sulfohydrolase; specifically desulfates chondroitin 4-sulfate	92
	Chondro-6-sulfatase	*Proteus vulgaris*	3.1.6.10	Sulfohydrolase; specifically desulfates chondroitin 6-sulfate	92
II	Testicular hyaluronidase	Bovine or sheep testes	2.2.1.35	Endo-β-N-acetylglucosaminidase; degrades hyaluronic acid and chondroitin sulfates	8
	Leech Head hyaluronidase	Leech Head	3.2.1.36	Endo-β-glucuronidase; degrades hyaluronic acid and chondroitin sulfates	8
	Chondroitin AC-lyase	*Arthrobacter aurescens*	4.2.2.5	Degrades chondroitin 4-sulfate, chondroitin 6-sulfate and hyaluronic acid	89
	Chondroitin ABC lyase	*Proteus vulgaris*	4.2.2.4	Degrades chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate and hyaluronic acid	89
III	DNAase I	Beef pancreas	3.1.4.5	Phosphodiesterase; specifically degrades most forms of DNA; used in sample preparation	17
	Ribonuclease A	Beef pancreas	2.7.7.16	Phosphodiesterase; specifically degrades most forms of RNA; used in sample preparation	17
	Papain	Papaya latex	3.4.4.10	Broad spectrum proteolytic activity; used in sample preparation	17
	Proteinase K	*Tritirachium album*	3.4.21.14	Broad spectrum proteolytic activity; used in sample preparation	17
	Clostripain	*Clostridium histolyticum*	3.4.22.8	Proteolytic activity; used in sample preparation	17
	Lipoprotein lipase	Bovine milk	3.1.1.34	Used in separation protocols as an affinity binding agent for heparan sulfate	115

of the unsaturated disaccharides produced by chondroitinase [71]. Likewise there are two distinct sulfotransferases which catalyze the transfer of sulfate from phosphoadenylsulfate to either the 4- or 6-positions of desulfated chondroitin or chondroitin oligosaccharides [73–75]. Human urine contains an endo-β-galactosidase specific for polylactosaminoglycan regardless of sulfation [76]. Another endo-β-galactosidase specific for the non-sulfated residues of keratan sulfate has been purified from *Pseudomonas* and is commercially available from ICN ImmunoBiologicals (Lisle, IL, U.S.A.) [77]. Thus keratan sulfate, like hyaluronate, may be specifically identified through the use of a single enzyme. Analogous to the chondroitin sulfotransferases there is a pair of phosphoadenylsulfate–keratan sulfate sulfotransferases which have been purified from bovine cornea cells [78]. Two forms of α-L-iduronidase have been identified. One of them specifically degrades heparan sulfate and not dermatan sulfate, while the other does just the opposite [79]. A specific heparitinase which degrades only heparan sulfate has also been purified from *Flavobacterium heparinum* [80].

4.3. Column liquid chromatography

4.3.1. Ion-exchange procedures

Conventional ion-exchange chromatography of GAGs is conducted chiefly with a DEAE ligand. Binding occurs at low ionic strength through carboxyl and sulfate charges. Separation of distinct GAG fractions is possible due to differing degrees of sulfation. It is also possible to separate the majority of glycopeptides since their charge density is proportionately less resulting in elution at low salt concentrations. Most commercial DEAE preparations, including DE52 (Whatman), DEAE-Sephacel and DEAE-Sephadex (Pharmacia Fine Chemicals) and DEAE-cellulose (generic), have been used and it is their similarities rather than their differences that stand out. The columns are ordinarily loaded in low salt (20–50 m*M*) at pH 7.0–7.4 in Tris or zwitterionic buffers. Urea up to 8.0 *M* is well tolerated and can be used to reduce the initial binding of many proteins [81]. However, urea may also inhibit the binding of desired proteoglycans and for that reason it is probably best omitted. The elution, usually performed with a sodium chloride gradient, follows a very characteristic pattern. For example, with rat liver homogenate, the first GAG to elute as the salt gradient is increased is hyaluronate. This occurs at about 0.45 *M* sodium chloride, and for some tissues may occur in conjunction with a high-molecular-mass heparan sulfate fraction. Heparan sulfate and a fraction of dermatan sulfate are eluted next, at about 0.65 *M* sodium chloride. Finally, a clean fraction of dermatan sulfate is eluted at 1 *M* sodium chloride [82–85]. In an alternative tissue, cultured embryonic mouse teeth, which possess a high content of chondroitin sulfate, a similar elution pattern is observed, but chondroitin replaces dermatan [86]. By adjusting buffer and running conditions, many researchers have reported essentially clean GAG separations.

4.3.2. Gel permeation chromatography

Due to their extreme size, high-molecular-mass polysaccharides may be purified by conventional preparative gel permeation chromatography. Molecular

sieving of GAGs is generally performed under dissociating conditions. The most frequently used gels are Sepharose 2B, 4B, 6B and the CL versions of each, as well as G-75 and G-100 (all from Pharmacia). The running buffers are commonly 0.5 *M* in sodium acetate and may contain other chaotropic ingredients such as 0.2 *M* sodium sulfate or 4.0 *M* guanidine hydrochloride. These chaotropic agents are included to avoid proteoglycan aggregates which will not separate by gel permeation. The use of gel permeation chromatography for the determination of molecular size has been largely replaced by high-performance procedures. Molecular sizing by itself is not routine, although it is possible with some of the gel matrices available. For example, Sephacryl S500 has an exclusion limit of 10 million, which is sufficiently large to permit the entry of the high-molecular-mass chondroitin sulfate proteoglycans through the gel pores [84]. A problem with the technique seems to be very broad peaks resulting in difficult separations and low resolution.

4.3.3. Hydrophobic interaction chromatography

Phenyl-Sepharose CL-4B (Pharmacia) has also been used for the separation of GAGs [87]. This hydrophobic gel acts by initially adsorbing the GAGs in the presence of high ionic strength (approximately 3.0 *M* ammonium sulfate) and eluting with an inverse salt gradient going towards water. It would appear that the retention of GAGs is closely correlated with their solubility in ammonium sulfate solution. A moderate differential solubility exists for hyaluronate, chondroitin and dermatan, which may be related to their molecular mass. Since the eluting salt concentration is typically greater than 1.5–2.0 *M*, it is reasonable to expect that a similar hydrophobic gel, octyl-Sepharose CL-4B (Pharmacia), which binds even more firmly at low ionic strength, would also be suitable for the separation of GAGs.

4.3.4. High-performance procedures

General HPLC GAG methods can be divided into two distinct groups. The first is separation and detection of constituent disaccharides produced by the use of enzymes. Hyaluronate, chondroitin 4-sulfate, dermatan sulfate and chondroitin 6-sulfate can be unequivocally identified by their characteristic disaccharides. The second HPLC methodology is concerned with separation of intact GAGs and proteoglycans.

4.3.4.1. Disaccharide separation. The separation and detection of constituent disaccharides is only useful if one can deduce from the monomeric structures those GAGs which must have been initially present. This deduction relies tremendously on the availability and specificity of degradative enzymes, mostly chondroitinase ABC and chondroitinase AC. Chondroitinase ABC acts on hyaluronate, chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate, whether alone or in proteoglycans, to reduce the glycosaminoglycan to Δ-4,5-unsaturated disaccharides (expressed as the unsaturated uronic acid component followed by the N-acetyl sugar component). The double bond coincidentally imparts to the molecule an absorption at 232 nm which is a convenient assay property. Chondroitinase AC acts essentially the same as ABC except that chondroitinase AC

cannot act on the iduronic acid of dermatan sulfate. To connect the old and new terminology, chondroitin 4-sulfate, dermatan sulfate and chondroitin 6-sulfate are chondroitin A, B and C, respectively. Thus, the basic premise for disaccharide constituent analysis may be stated as follows: (1) 4-sulfated disaccharide produced by chondroitinase AC must have derived from chondroitin 4-sulfate; (2) 6-sulfated disaccharide produced by chondroitinase AC must have derived from chondroitin 6-sulfate; (3) the dermatan sulfate component is best estimated by the difference in total disaccharide produced by the separate action of both enzymes. Because considerable heterogeneity in the primary structure exists, extreme care must be exercised in composition analysis based on disaccharide data alone. Dermatan sulfate by definition is a chondroitin sulfate with a predominate, but not exclusive, composition of iduronic acid rather than glucuronic acid. The disaccharide analyses alone cannot be used to identify those condroitin-like sequences found in dermatan molecules, and the tendency is therefore to underestimate the total dermatan sulfate.

The preparation for disaccharide analysis of biological samples, such as urinary GAG, requires initial purification work. Since the detection system is UV absorbance, interfering substances must be removed. A suggested scheme is serial DNAase I/protease addition to reduce the size and increase the solubility of the protein and DNA fractions. This may be followed by cetylpyridinium chloride precipitation of the GAGs. Solubilization of the cetylpyridinium precipitate followed by reprecipitation with ethanol will reduce the cetylpyridinium contaminant. Solubilization of the ethanol precipitate followed by hyaluronate lyase treatment will specifically produce small hyaluronate fragments. These fragments, as well as residual cetylpyridinium, may be removed on a desalting column. Keratan sulfate, due to its small size, is also lost by this procedure. The remaining GAG fraction should be primarily chondroitin, dermatan and heparan sulfates. The residual GAG may now be digested with chondroitinase to obtain the constituent disaccharides. It should be noted that losses occur in these precipitation steps and thus quantitation should ultimately be presented as percentages of total disaccharide.

A simple reliable method for HPLC separation of the unsaturated disaccharides produced from chondroitin and dermatan sulfate was described by Fluharty et al. [88]. It uses a standard silica (Partisil 10 XAS) column (25 cm) and an isocratic 7.5 m*M* potassium phosphate buffer elution for the first 7 min followed by a gradient elution rising to 500 m*M* phosphate, all at pH 6.5. The higher salt is required to remove multisulfated disaccharides. A similar silica gel (Partisil 10 PAC) system has been described using a completely isocratic ternary solvent, acetonitrile–methanol–ammonium formate. Using sodium borohydride to reduce the disaccharide carbonyls to terminal hydroxymethyl groups eliminates α,β-anomeric forms of the sugars, with subsequent improvement of peak resolution [89]. The sensitivity of the method may be improved into the high picomole range by the use of a fluorescent derivatization produced by coupling 2-aminopyridine to the unsaturated disaccharide with no effect on elution order of the disaccharides [90]. The resultant pyridylamino derivatives are detected using a fluorescence spectrophotometer with excitation and emission at 310 and 375 nm,

respectively. A true alternative to silica, which has been used quite successfully in these systems, is a sulfonized styrene–divinylbenzene copolymer (Shodex RS Type DC-613 from Showa Denko, Tokyo, Japan) [91]. With this system the order of peak elution is changed, but separation comparable to the silica matrices is achieved using an isocratic solvent elution (acetonitrile–methanol–0.5 *M* ammonium formate, 65:15:30, v/v/v, pH 4.5). Resolution appears to be improved without the need of hexose reduction by borohydride.

In that the chondroitin and dermatan sulfates are not uniformly sulfated, it is common to obtain peaks, which correspond to the disulfated configuration. Using the Shodex RS column and the purified enzymes, chondro-4-sulfatase and chondro-6-sulfatase, Murata and Yokoyama [92] have furthered disaccharide analysis by identifying which peaks correspond to precisely which sulfated forms. This technique now offers greatly increased opportunity to study the heterogeneity within the chondroitin–dermatan glycans.

When hyaluronate is acted on by chondroitinase, one merely obtains non-sulfated Δ-4,5 disaccharides. However, Streptomyces hyaluronidase will specifically degrade hyaluronate to tetra- and hexasaccharides which are also Δ-4,5-unsaturated. Silica columns may be used to separate these components and thereby quantitate the hyaluronate fraction of a GAG sample [93]. The solvent is again acetonitrile–methanol–0.5 *M* ammonium formate (10:6:3, v/v/v, pH 6.0) used isocratically at room temperature. In order to use this procedure in determining the hyaluronate concentration of fluid samples (e.g. synovial fluid or vitreous) one might first incubate and exhaustively degrade the fluid with hyaluronate lyase and then ethanol precipitate to remove the remaining protein, nucleic acid and non-hyaluronate GAG "contaminants". The hyaluronate oligosaccharides may then be recovered from the ethanol supernatant by evaporation.

4.3.4.2. Polysaccharide separation. Due to the development of improved gel matrices and commercial packing of liquid chromatography columns, it is now practical to separate and size differing molecular mass fractions of GAGs and proteoglycans using gel permeation HPLC. The principal advantage of these columns is the ability to rapidly examine the distribution of GAGs in biological fluids. Although several manufacturers are marketing columns for this application, it appears that only two gel matrix types are made. One is a glycerylpropylsilane hydrophilic phase bonded to 10 μm diameter silica particles. Multiple pore sizes are possible but the useful working range is 10–400 nm. For example, Brownlee Labs. produces OH-500 and OH-1000 Aquapore, and SynChrom makes SynChropak [94,95] both of which are comprised of this gel type. Toyo Soda produces TSK G6000 PW and TSK G5000 PW, which are described as being a cross-linked hydrophilic polymer phase bonded to a silica support. The other matrix used for this application is the hydrophilic organic polymer poly(hydroxyalkyl methacrylate) produced by Showa Denko and sold as Shodex OH Pak 800pR.

These columns are a success. They permit adequate and quick separation of the high-molecular-mass hyaluronate, nucleic acid and proteoglycan fractions of unprocessed biological fluids from the overwhelming number of smaller proteins. Any aqueous buffer may be used which is compatible with the method of detec-

tion. Low ionic strength (0–0.6 *M* salts) may offer slightly better resolution. The pH is best kept between 3 and 7, but may be varied to the extremes for a short time without significant deterioration of the matrices. Thus, between runs, adsorbed proteins may be effectively washed off with high salt at high pH followed by equilibration of the gel with a buffering solvent. Non-aqueous solvents may also be used, but they do not appear to offer any advantage and are generally incompatible with biological samples.

Hyaluronic acid and proteoglycan separations are the two primary targets for these high-performance columns. The other non-associated GAGs are of sufficiently low molecular mass as to be easily tractable by older technology. Hyaluronic acid is readily separated away from the proteins and DNA of most biological fluid by the G6000 PW column. Tandem columns offer increased resolving power, but for most work a single 30 cm×7.5 mm column is adequate. DNA, if present, may be degraded with DNAase I, followed by protease digestion and trichloroacetic acid precipitation of protein. If protease is not to be used, then multiple columns are a requirement when applying crude biological fluids such as synovial fluid or the aqueous of the eye. These untreated fluids contain numerous compounds which bind transiently to the column and are eluted well after the solvent peak. A guard column is mandatory to protect the analytical column.

Purified hyaluronate exogenously added to crude samples is quantitatively recovered. Hyaluronate size within these biological fluids may be determined by comparison to polyethylene oxide standards [96]. The interaction of proteoglycan subunits with hyaluronate is apparently dissociated by the shear forces encountered during the chromatography. As a result, G6000 PW is not useful for the study of proteoglycan aggregation, even when link protein is exogenously added [97].

The Aquapore glycerylpropylsilane-silica columns may prove to be superior in the study of untreated biological samples. This matrix is capable of separating individual proteoglycan monomers from lower-molecular-mass GAGs and proteins. Large proteoglycan aggregates are also passed through the column without apparent degradation as judged by post-column electronmicroscopy [98]. While the Aquapore matrix has been used to characterize newly synthesized proteoglycans from rat embryonic parietal yolk sac [99], the Shodex poly(hydroxyalkyl methacrylate) matrix has been successfully used only with purified chondroitin sulfate and hyaluronate samples of molecular mass less than 1 million [100]. With this latter matrix the questions of proteoglycan aggregation and upper limit of molecular size have yet to be addressed.

4.3.4.3. Molecular mass standardization. Suitable calibration standards for HPLC columns are not readily available. Traditionally, a few high-molecular-mass protein standards and well characterized dextrans have been used for the standardization of size exclusion chromatography columns. Such molecular-mass standards are not useful when the included volume of the proposed column is capable of accepting water-soluble cellulosics with molecular masses approaching 10 million. Proteins are reasonably well defined standards but they are too small to be used in large pore matrices. Polymers, such as dextran, are far less defined and must be fractionated and calibrated one batch at a time. Furthermore, dif-

ferent methods of molecular-mass determination invariably result in different estimations of size. Indeed, the size of the polymers, which constitutes the primary measurement, is highly affected by the solvent conditions. Still, heterogeneous polymers are the primary molecular-mass standards because no other suitably sized molecules are available. Besides dextrans, characterized mainly by Pharmacia (Uppsala, Sweden), there are polyethylene oxides (Toyo Soda) and polyacrylic acids (Modchrom, Mentor, OH, USA), available as standardized molecular-mass kits. The polyacrylic acids in aqueous solvent do not elute properly from the G6000 PW columns. They appear to be retarded by adsorptive interactions independent of molecular sieving. Thus their elution profile, while sequential with molecular size, is demonstrably behind the small-molecule solvent front. Indeed, it has been noted by this author that many different molecules (mostly low-molecular-mass molecules) which contain an unsaturated bond or aromatic ring structure are retarded by the G6000 PW matrix in a manner inconsistent with molecular sieving. These observations are the result of highly reproducible migration times with elution two to three times that of the solvent front. The polyethylene oxide standard kit offered by Toyo Soda has an upper limit of 1 million. Higher-molecular-mass polyethylene oxides are readily available from the large chemical supply houses, but to be useful they must first be independently fractionated and sized. A potentially superior set of high-molecular-mass standards could be produced from deoxyribonucleic acid using restriction enzymes and electrophoretic techniques to accurately produce and characterize high-molecular-mass oligonucleotide sequences of defined tertiary conformation. Nucleic acids are well behaved in aqueous media on these gels. Currently, such kits are not commercially available.

4.4. Electrophoresis

4.4.1. Cellulose acetate procedures

Chain length and molecular size are somewhat less of a problem in cellulose acetate electrophoresis because the method has been optimized to separate GAGs according to charge density rather than absolute size. However, GAGs of uniform charge density having vastly differing molecular size populations, such as can be found with hyaluronate, may still result in heterogeneous streaks and smears. Separation of GAGs also suffers from the presence of the so called "copolymeric" structures which are not uniformly 4- or 6-sulfated, but are rather a seemingly random mixture of permissible primary structures. Again, poor resolution, marked by band smearing, is the result. Cellulose acetate electrophoresis remains a powerful tool due to the development of techniques which have improved resolving power.

Since endogenous GAGs exist as proteoglycans, biological tissue samples or fluids must be protease-treated to release the polysaccharides prior to electrophoresis. It is possible after only a protease treatment to separate hyaluronate from the sulfated GAGs. However, to obtain finer resolution the technique usually requires defatted, delipidated tissue which is sequentially protease-treated, desalted, lyophilized and redissolved. Any procedure which partially purifies the

GAG fraction of biological samples is a potentially suitable preparation for qualitative electrophoretic analysis [101]. While quantitation of individual GAG components can be achieved using cellulose acetate electrophoresis, care must be exercised in extrapolating these values back to the original tissue or fluid. Without the judicious use of internal reference standards during the sample isolation and purification steps, such back-calculations become meaningless.

The methodology for improving resolution requires GAG purification. This usually involves multiple steps and the yield at each step is always in question. An aliquot (ca. 1–20 μl) of the purified fraction is applied to a cellulose acetate strip and run, along with GAG standards, in an acidic buffer [102]. A suitable well resolved one-dimensional system [103] requires three electrophoretic steps, a 3-min electrophoresis followed by a 1-min buffer soak (0.1 *M* barium acetate, pH 5.0), an 8-min run followed by a 1-min soak in the buffer plus 18% (v/v) ethanol, and a final 20-min run. This system uses 6×7.5 cm sheets having a twelve-sample capacity. An equally well resolved two-dimensional system [104] utilizes two buffer systems. The first dimension is 1.0 *M* pyridine formate, pH 3.1, run at 20 mA for 70 min. The second dimension buffer is 0.1 *M* barium acetate run at 20 mA for 7 h. This two-dimensional system requires a 15×15 cm sheet of cellulose acetate for each sample. Alcian blue staining permits visualization of the most concentrated spots. Quantitation schemes may include densitometry or copper analysis of the cleared cellulose acetate strips [103,105] or UV absorbance or copper analysis of the dye following dissolution of the spot in dimethyl sulfoxide [106].

4.4.2. Polyacrylamide procedures

GAG oligosaccharides produced by the action of chondroitin AC lyase or testicular hyaluronidase may be separated by conventional polyacrylamide gel electrophoresis using a 15% gel and autoradiographic detection [107]. The basis for separation is primarily molecular size which is analogous to the separation of DNA oligonucleotides. However, the shortest oligosaccharides (less than ten monomers) appear to behave anomalously. The method may also be scaled up and used preparatively to obtain well defined fractions of oligosaccharides [108]. Such preparative oligosaccharide preparations produce sufficient quantities of discretely sized oligosaccharides for subsequent investigations of the epimerization process, protein binding properties and structural conformation.

A polyacrylamide agarose electrophoretic procedure has been developed which separates the major proteoglycans of cartilage [109,110]. By the inclusion of sodium dodecyl sulfate and Triton X-100, suitable dissociative conditions have been found such that even crude guanidine-hydrochloride extracts of cartilage tissue can be separated. In such systems smaller proteins run at the solvent front while the more massive proteoglycans separate into distinct bands. It is interesting to note that, of nine different cartilage tissues run by this technique, only three electrophoretic bands were observed. These were identified as: (1) a fast migrating band of small proteoglycans; (2) a keratan sulfate-rich proteoglycan; and (3) a slow migrating band of chondroitin sulfate-rich proteoglycans [110]. It has also been possible with this technique to visibly demonstrate the aggregate

association of these proteoglycans with hyaluronic acid. When hyaluronate is mixed with the proteoglycans prior to electrophoresis, the migration distance is immensely shortened, indicative of the higher-molecular-mass aggregate [110]. Detection is accomplished by either fluorography or Toluidine blue staining. This procedure undoubtedly has a potential application for the analysis of proteoglycan changes associated with disease states, affecting cartilage, synovial fluids, dermis or vitreous.

4.5. Gas chromatography

Gas chromatography has been used with mixed results for the determination of the GAG content of biological tissues [111–114]. Its primary use has been the identification of constituent monomeric sugars. As in disaccharide analysis with HPLC, an intact GAG fraction should first be purified to homogeneity by conventional means to assure an unambiguous assignment of the constituent monosaccharides. Aside from the concerns of sample purity, the difficulties involved in queantitative sample hydrolysis and derivatization preclude gas chromatography as the "method of choice" for constituent monosaccharide analysis of GAGs. Gas chromatographic methods are complicated by the need to cleave the GAG polymer into intact monosaccharides which can be suitably derivatized prior to chromatography. Aqueous acid hydrolysis of the polymer results in a loss of the very labile uronic acid residues. As such, only hexosamines and neutral monosaccharides are available for subsequent derivatization and separation. Methanolysis and trimethysilylation techniques are relatively ineffective since de-N-acetylation of the N-acetylhexosamine occurs. This produces a glucuronidic linkage to galactosamine which becomes resistant to methanolysis and inhibits further depolymerization. Alternative procedures involving combinations of deamination, methanolysis and re-N-acetylation steps have been proposed in an attempt to simultaneously analyze all of the constituent sugars of various GAGs. Overall, it appears that such extensive sample manipulation is not favored by the majority of investigators, an observation supported by the relative scarcity in this decade of GAG-related publications which have used gas chromatographic procedures.

4.6. Biospecific affinity chromatography

Since GAGs are known to aggregate or associate with proteins or other GAGs, these phenomena have been used to develop a variety of biospecific affinity chromatographic media. As yet, the nature of this binding has not been clearly resolved. Lipoprotein lipase has a general affinity for GAGs which decreases in the order heparin (strongest), heparan sulfate, dermatan sulfate and chondroitin sulfate (weakest) [115]. Lipoprotein lipase, purified from fresh skim milk by heparin–agarose affinity chromatography, may be coupled to cyanogen bromide-activated Sepharose-4B to produce a heparan sulfate affinity column [116] capable of purifying heparan sulfate-rich proteoglycans in the presence of chondroitin sulfates. The basement membrane glycoprotein, laminin, has also been reported

to have a high affinity for GAGs [117], and a laminin affinity matrix has been prepared from glomerular basement membrane [118]. While hyaluronate has an affinity for chondroitin 6-sulfate [119] its affinity for the protein portion of proteoglycan may be much greater.

Cartilage proteins with a high affinity for hyaluronate are collectively known as link protein and are currently thought to cause aggregation of proteoglycan and hyaluronate. Coupling hyaluronate to AH-Sepharose-4B has become a relatively common procedure for the purpose of purifying these HA-binding proteins from a variety of sources [120–122]. Briefly, the procedure from cartilage involves extraction of the GAG with 4.0 *M* quanidine hydrochloride, followed by dialysis and lyophilization of the supernatant. The lyophilized powder is redissolved and partially trypsinized, dialyzed versus 4.0 *M* quanidine hydrochloride and then dialyzed again with the HA-derivatized gel versus distilled water. The link proteins are then eluted from the affinity gel with 4.0 *M* guanidine hydrochloride and 0.5 *M* sodium acetate, pH 5.8.

4.7. Discussion of individual glycosaminoglycans and specific separation procedures

Much effort within the research community has gone into locating, identifying and quantitating GAGs. Although they are ubiquitous within membranes, cells, matrix material and organ systems of mammals, different tissues and sources possess very different GAG compositions. A separate discussion follows about each of the GAGs prior to considering them collectively as proteoglycans.

4.7.1. Hyaluronate analysis

Sodium hyaluronate occurs in cell coats, extracellular ground substance of connective tissues, synovial fluid, vitreous humor, umbilical cord, dermis, aortal flap and numerous other locations. Purification schemes have evolved slowly, starting with Boas [123] and others [124–126] in the late 1940s and continuing through today. Scott [5,6] probably contributed the most to the development of purification schemes with a thorough examination of the variables involved in precipitation by aliphatic ammonium salts. Due to the commercial importance of hyaluronate as a vitreous replacement fluid and as a joint replacement fluid, its water retention properties and, therefore, its physical size have become very important factors. Size-exclusion chromatography has proven reliable for commercial quality control of sodium hyaluronate products [96,97,100,127]. However, the currently available technology is only marginally able to size the largest hyaluronate molecules. Furthermore, based upon current levels of interest within the scientific community, a breakthrough in the ability to size large, negatively charged molecules may well occur first in the field of nucleic acid polymer chemistry.

Biospecific affinity chromatography has been used for the purification of hyaluronate although the technique is not common. Simian virus 40 transformed cells (SV-3T3) possess on their cell surface high-affinity binding sites for high-molecular-mass hyaluronate [128,129], and an affinity chromatography column has been prepared which utilizes these sites after glutaraldehyde fixation of the

cell [130]. Cartilage proteoglycans also possess a very high affinity for hyaluronate. By attaching rhodamine to the core protein portion, these proteoglycans have been used both as a probe for cell surface hyaluronate distribution [131] and as an affinity ligand [120].

Despite a vast amount of research with hyaluronate, especially within the pharmaceutical community, the routine measurement of concentration within biological tissues has remained difficult. For concentrations of 100 μg/ml and higher, the HPLC assay of Beaty et al. [96] is very convenient, although controls are required to assure that hyaluronate and not nucleic acid or proteoglycan aggregate is being measured. For urine, serum or amniotic fluid which may contain less than 100 μg/ml hyaluronate, one currently must move to the more involved radioassay [120,132–134]. This assay requires purification of the globular hyaluronate binding region of the proteoglycan protein core and link protein from bovine nasal cartilage. Although very sensitive, this assay is not trivial to perform.

In all of the available quantitative assays, sample preparation must be minimized. Protease or nuclease addition is permissible and well tolerated. Lipid extraction by chloroform is acceptable, but precipitations using alcohol, acetone or quaternary ammonium salts should be excluded, as they cannot be performed quantitatively. The carbazole (uronic acid) or hexosamine assays are notoriously imprecise. For example, although glucuronolactone is the usual standard for the carbazole assay, use of glucuronic acid will yield totally different results when compared on a mole-for-mole basis in the same assay. Commercial preparations of hyaluronate powder are impure and hydrated, containing 2–15% moisture. Pharmaceutical injections of sodium hyaluronate are the purest commercially available preparations. However, even the pharmaceutical "standard" Healon (Pharmacia) possesses a 260-nm absorbance of 0.2–1.0, whereas pure hyaluronate has no absorbance in that spectral region. Therefore, in critical research applications the prudent investigator should independently establish the purity of any commercial hyaluronate preparation.

4.7.2. Analysis of chondroitin and dermatan sulfates

The advent of monoclonal antibodies may shortly be expected to add new specific assays for each of the GAGs. For example, an antibody has been raised against the chondroitinase ABC-digested bovine nasal cartilage proteoglycan which recognizes the unsaturated uronic acid residue linked to N-acetylgalactosamine 4-sulfate [135]. One can easily imagine the chondroitin 4-sulfate assay which this suggests, and indeed it has been used to demonstrate the complete absence of chondroitin 4-sulfate in the epidermis around invaginating hair follicles [136]. A monoclonal antibody to a dermatan sulfate proteoglycan has also been developed [53] but it is not clear what portion of the molecule carries the antigenic determinant. Such probes represent the best available technology for monitoring the presence or tracking the purification of a specific proteoglycan.

The HPLC disaccharide assay of Murata and Yokoyama [91,92] provides the best available tool for further study of heterogeneity within the chondroitins. Their method (sulfonized styrene–divinylbenzene copolymer) resolves all of the possible sulfated disaccharide isomers. Discrete oligosaccharide components of

chondroitinase-digested chondroitin sulfates have also been resolved by polysaccharide gel electrophoresis [84].

4.7.3. Keratan sulfate analysis

The development of monoclonal antibodies has significantly advanced the study of keratan sulfate. Caterson et al. [54] have developed an antibody, "1/20/5-D-4", of the immunoglobulin G_1 (IgG_1) classification which appears to be specific for the polysaccharide portion of keratan sulfate. Their evidence suggesting specificity is that skeletal keratan sulfate and corneal keratan sulfate, which are known to have different peptide linkages, yield very similar inhibition curves in a radioimmunoassay. Also, removal of the sulfur from corneal keratan sulfate produced a significant reduction in recognition by the antibody. Specifity of the monoclonal antibody was also demonstrated by a lack of antibody inhibition in the presence of dermatan, heparan sulfate, heparin, hyaluronate and rat chondrosarcoma proteoglycans which are specifically known to lack keratan sulfate. Recently, two other keratan sulfate monoclonal antibodies have been produced (1-B-4 and MZ15) and continued analysis by radioimmunoassay confirms that the specificity for all three antibodies is directed against the sulfated poly(N-acetyllactosamine) sequences [137]. Non-sulfated sequences obtained from glycoproteins and desulfated keratan sulfates or desulfated keratan sulfate hexasaccharides are not recognized by the antibodies. The minimum sequence length required for full binding varied slightly among the three antibodies but the pentasulfated hexasaccharide was the smallest oligosaccharide which bound all three. As one might expect, the immunosorbant inhibition assay can be used to obtain clinically relevant information about the presence of keratan sulfate in various disease states. Patients with macular corneal dystrophy exhibit no serum keratan sulfate while patients with osteoarthritis have significantly high serum levels [138].

4.7.4. Heparan sulfate analysis

Heparan sulfate associated as proteoglycan is the major GAG of the glomerular basement membrane and is ever present, as well, in plasma membrane. It is believed to play a very important role in both membrane permeability and intercellular communication [139]. However, much of the research effort has concerned the role of heparan sulfate proteoglycans in brain and nerve tissue. As with the other GAGs, monoclonal antibodies have been developed for use with radioimmune inhibition assays and these have been particularly helpful in the localization of heparan sulfate proteoglycans during various stages of development [140–146]. Additionally, an affinity chromatography system has been developed which uses lipoprotein lipase bound to agarose as the affinity medium. This system permits separation of heparan sulfate proteoglycan from the more voluminous chondroitin fractions [115].

4.7.5. Proteoglycan separation and analysis

The GAGs associated into proteoglycan aggregates are ubiquitous as part of the extracellular matrix. Quantitation is difficult because aqueous extraction is

not easily achieved. As a result, the conditions of extraction become quite important to all reports of a new proteoglycan. Research efforts have been focused primarily on three specific sources, bovine nasal cartilage, bovine tracheal cartilage and rat chondrosarcoma cells. Most of these proteoglycans associate into very large aggregates. As such, the use of molecular sieving chromatography as it is currently understood would be ludicrous. For example, if an aggregate consists of 100 proteoglycan molecules each of about 2.5 million and one hyaluronate sequence (2 to 5 million – negligible!), the total aggregate molecular mass is 250 million. Successful extraction procedures disrupt the aggregate and rely upon diffusion to solubilize the molecules. Some procedures (4.0 *M* quanidine hydrochloride at 4°C for several days) are more gentle than others (mechanical grinding followed sequentially by quanidine hydrochloride and protease for several days). The end result is a solution of proteoglycan or smaller molecules devoid of aggregates. It is obvious that protease treatment to facilitate proteoglycan release may result in an underestimate of the proteoglycan molecular mass. Consequently, one may be left with comparisons of individual GAGs and merely inference about the proteoglycan.

The chromatographic and electrophoretic methods by which one can study the proteoglycans are principally those which have already been discussed. The components of the proteoglycan aggregate must be torn apart and inspected with the hope that a comparison between the disease and normal states will reveal consistent differences. One of the methods by which extracted proteoglycans may be rapidly separated from other proteins is through the use of a cesium chloride density gradient produced in a small air-driven ultracentrifuge [147]. The procedure requires a very small sample size and can be performed on multiple samples simultaneously, thus permitting direct comparison of identically purified proteoglycan fractions from normal and disease affected cases. Multiple samples are also of great benefit in the acquisition of statistics, a necessary nuisance in the quantification of human data.

For larger preparative samples, DEAE-cellulose chromatography is the primary method for separating proteoglycans from glycoproteins. The unbound glycoprotein fraction is washed through the column while the bound proteoglycans are easily eluted at higher salt concentrations. Through the use of lyase enzymes, enriched fractions of specific GAG-containing proteoglycans may be obtained. For example, in the presence of protease inhibitors and chondroitinase ABC, the monkey cornea proteoglycan fraction is composed exclusively of keratan and heparan sulfates. These may be further separated on ConA-Sepharose, which binds only the keratan sulfate proteoglycan and can be eluted, with methylmannoside [148]. An alternative approach for the purification of an enriched heparan sulfate fraction is to use a lipoprotein lipase affinity ligand and elute the column with an increasing sodium chloride concentration [115].

When available, monoclonal antibodies offer the best available technology for the identification of either specific proteoglycans or classes of GAGs. An excellent general discussion of the production of monoclonal antibodies directed against connective tissue proteoglycans has been written by Caterson et al. [149]. They also describe the production of six antibodies and discuss their specificities. In

summary, these include antibodies to (a) the disaccharide keratan sulfate, (b) the unsaturated or saturated tetrasaccharides derived from chondroitin 4- or chondroitin 6-sulfate and (c) the unsaturated disaccharides of chondroitin or hyaluronic acid. New specific monoclonal antibodies continue to be developed.

Basically, there are two HPLC methods for studying proteoglycans. These are disaccharide analysis and molecular sieving. Ion-exchange columns using a binary salt gradient elution are feasible, but have not thus far been applied. Disaccharide analysis following specific lyase digestion [88,89,150,151] has the advantage of relative quantitation of the so called "co-polymeric" substructures of the specific classes of GAGs. For example, within the class chondroitin sulfate one has type A (4-SO_4^{2-}), B (dermatan), disulfated B (uronic 2-SO_4^{2-}, 6-SO_4^{2-}), C (6-SO_4^{2-}), D (uronic 2-SO_4^{2-}, 6-SO_4^{2-}), E (4-SO_4^{2-}, 6-SO_4^{2-}), H (dermatan 4-SO_4^{2-}, 6-SO_4^{2-}), K (uronic 3-SO_4^{2-}, 4-SO_4^{2-}), and trisulfated dermatan (uronic 2-SO_4^{2-}, 4-SO_4^{2-}, 6-SO_4^{2-}). Whether or not the increased information gained through the use of this system is worthwhile depends on the medical relevance of the particular study. The other HPLC method, molecular sieving, has limited resolving power for proteoglycans unless numerous columns are run sequentially, which translates into lengthy run times [97–99]. The principal advantage of gel permeation is that, with the use of specific lyase digestion, naturally enriched GAG fractions from biological fluids or tissue extracts may be easily quantitated. Thus, a kinetic study which observes the concentration of a GAG or proteoglycan can be performed to monitor the progress of a disease.

Electrophoresis of intact proteoglycans remains a new technology [110]. The technique employed (agarose polyacrylamide) is analogous to the separation of large DNA sequences. Thus far, the resolving power is no better than that of gel permeation HPLC and the quantitation must be considered more difficult.

Proteoglycans also have a protein component, and while that is not the subject of this review, it is prudent to mention that some excellent work has been performed on the purification and characterization of core proteins [152–154]. At present, the most interesting feature of the core protein is the hyaluronate binding region, which has been shown to possess less polysaccharide than the remainder of the protein. Using the aggregating proteoglycan of the swarm rat chondrosarcoma it has been possible to locate the hyaluronate binding region on the N-terminus of the protein, a region possessing few or no chondroitin sulfate chains [153].

Link protein describes any glycoprotein (not proteoglycan) which has an increased affinity for hyaluronate and which promotes the aggregation of proteoglycans and hyaluronate. Measurements of aggregate size are performed in analytical ultracentrifuge sedimentation velocity experiments. Purification of link protein is as varied as protein purification in general [155]. However, biospecific affinity chromatography is the most fequently used method. Since link protein is known to have an affinity for hyaluronate, the glycoprotein may be further fractionated on hyaluronate-Sepharose using 3.0 *M* thiocyanate as eluent [156,157]. Link protein also has an affinity for lectins and wheat germ agglutinin has been used as an affinity ligand with N-acetylglucosamine as eluent [158].

5. GLYCOSAMINOGLYCAN ASSOCIATED DISEASES

5.1. Mucopolysaccharidosis

The obvious diseases associated with GAGs are the mucopolysaccharidoses. The sufferers of this group are often phenotypically grotesque and mentally retarded. Except for the possibility of bone marrow transplantation [159,160], there is no cure nor effective treatment. Since the diseases are inherited, genetic counselling may prevent future births of affected individuals [161]. A thorough review of the individual diseases, all of which are catabolic enzyme deficiencies, is available [162].

Phenotypic diagnosis of mucopolysaccharidosis is followed by laboratory screening of urinary GAGs using a wide variety of methods. These tests are performed at three levels. First, a paper spot test [163] or quaternary ammonium turbidity test [164] is used to identify greater than normal urinary GAG. Second, specific testing is performed to identify the relative concentrations of sulfated GAG, thereby suggesting which defects might actually be present. Third, the demonstration of deficiencies of specific lysosomal enzymes normally responsible for catabolism of the various GAGs confirms the diagnosis.

A critique of the cellulose acetate electrophoresis method for urinary GAG determination and a statistical analysis of the Ames paper spot test versus the cetylpyridinium chloride–citrate turbidity test has been reported by Huang et al. [165]. They concluded that the spot test was superior for identification of urinary GAG, yielding only 2.9% false positives and 3.4% false negatives, and if the urine concentration was adjusted (or corrected) to 10 μmol creatinine then zero false results could be obtained. The second step, screening of urine samples for GAG patterns, can either be done electrophoretically [166–168] or by HPLC [169–171]. The HPLC method involves disaccharide analysis, as previously described, on a Partisil 10 PAC (10 μm, 25 cm $\times$ 4.6 cm) column. Whether electrophoresis or HPLC analysis is used, the urinary GAGs must be isolated by cetylpyridinium chloride precipitation and partially purified, requiring an effort of one to two days. A method sufficiently sensitive to permit a more direct analysis from urine is needed. Urine concentrations of uronic acid in these diseases are estimated at 0.02–0.2 mg/ml, nearly within the range of the available assays. For example, the Coomassie blue–BSA–GAG staining technique for cellulose acetate strips has a reported detection of 15–40 ng of GAG per 1-μl spot [43].

5.2. Periodontal disease

In addition to mucopolysaccharidoses, changes in GAGs are important in periodontal disease, osteoarthritis, rheumatoid arthritis and other less prevalent anomalies. In periodontitis it is apparent that sulfated GAGs serve as a marker of tissue breakdown. Hyaluronate is a normal component of gingival crevicular fluid, while chondroitin 4-sulfate is a characteristic of untreated chronic periodontal disease involving the deeper periodontal tissues. The concentration of chondroitin 4-sulfate in these tissues is sufficient to permit direct application of

crevicular fluid to cellulose acetate for electrophoresis. Indeed the appearance of chondroitin 4-sulfate in the gingival crevicular fluid would appear to differentiate gingivitis, where inflammatory changes are confined to the soft tissues, from periodontitis, where destructive activity in the deeper tissues is present [172–178]. Proteoglycans are also isolated from dental pulp and dentine, and the GAG composition of these tissues exhibits distinct differences [179]. Indeed, an HPLC analysis of puppy dentine consisted primarily of chondroitin 4-sulfate and chondroitin 6-sulfate while hyaluronate, keratan or heparan, even in trace amounts, were not present [180,181].

5.3. Osteoarthritis

The HPLC techniques are especially pertinent to the research of disease processes which compare differences in healthy and diseased tissues. The identification of specific GAGs is of interest because of a widely held belief that several common chronic disease processes should produce biochemical changes in either the primary structure or the relative proportions of certain affected GAGs. A case in point is arthritis. We know that arthritis eventually results in near total degeneration of the articular surfaces and we further know that in the laboratory, working with healthy articular cartilage, the conditions for extraction of proteoglycan result in severe cartilage degeneration. Yet those extreme conditions (approximately 4.0 *M* guanidine hydrochloride) are never encountered in vivo. Thus in arthritis, one can reason that fundamental biochemical changes must occur which are devastating to the intercellular matrix of cartilage, and by association, to the constituent GAGs. It would seem logical that close examination of the composition and primary structure of GAGs and proteoglycans in both healthy and diseased tissues will reveal those fundamental changes.

The monoclonal antibodies, which have been developed to react with specific structural features of cartilage proteoglycans, provide the best opportunity to study the arthritic disease process [149,182]. The most significant finding in this area has been the coincidental increase of serum keratan sulfate levels with confirmed osteoarthritis [138]. This finding is a result of the development of monoclonal antibodies to keratan sulfate. Age-related differences in serum keratan sulfate exist between children and adults, but among adults no significant age-related differences were observed. The mean osteoarthritic serum level was 357 ± 73 ng/ml, while normals were 251 ± 78 ng/ml. As judged by DEAE and Sepharose CL-6B chromatography, the excess serum keratan sulfate was free GAG rather than proteoglycan. Thus far, no study exists to observe changes which might occur in the progression of the disease. However, this method could potentially be very useful in the early diagnosis of joint disease.

Animal models of osteoarthritis are useful since onset of the disease can be hastened and experimental parameters can be better controlled. Using a strain of osteoarthritic mice, Rostand et al. [147,183] have shown that osteoarthritic articular cartilage proteoglycans are very similar to normals with respect to molecular size and degree of sulfation. However, the osteoarthritic cartilage yields significantly more proteoglycan than normals when extracted with 4.0 *M* guani-

dine hydrochloride. Osteoarthritis may be produced similarly in the dog stifle joint by transection of the anterior cruciate ligament. In contrast to the mouse, the dog model has shown that osteoarthritic proteoglycans are larger than normals, due to the presence of abnormally long chondroitin sulfate chains on newly synthesized proteoglycans. These results were obtained through gel permeation chromatography with Sepharose CL-2B, -4B and -6B [184]. A rabbit model, similar to the dog model, indicated an increase in total GAG and a decrease in cartilage keratan sulfate as shown by disaccharide analysis [185]. Yet another model which has been investigated calls for the injection of glucose oxidase into the joint. This enzyme produces hydrogen peroxide within the joint space and results in histological damage similar to that observed with advanced osteoarthritis [186]. Despite the occurrence of arthritic processes in animal models, results continue to reflect only the chronic aspects of the disease. A predictable acute phase arthritic model is still needed.

In addition to mammalian in vivo models, there are many studies of primary chondrocytes in cell culture. The advantages of cell culture are still greater biochemical control, as opposed to in vivo models, and the convenience provided by a non-animal system. One of the potential defects in osteoarthritis is increased proteolysis of GAG. Proteoglycan characterizations using primary chondrocyte cultures, and Biogel P-60 and Sepharose CL-2B chromatography have indicated that specific metalloproteases play a role in the normal metabolism of proteoglycans [187]. Conversely, another potential defect is in the synthesis of GAG. Organ culture of osteochondrophytic spurs of human femoral heads have been used to study in vitro proteoglycan biosynthesis [188]. The value of these models is in rapid experimentation which hopefully will lead to testable hypotheses in human osteoarthritis.

Studies of normal versus naturally occurring osteoarthritic populations in which the size of proteoglycans and GAGs or the activity of cartilage protease fractions were observed have been able to demonstrate few significant differences. For example, human urine measurements of total GAG show no change [189]. Bovine cartilage proteoglycan and GAG chromatographic size are not different [190], and guinea pig cartilage proteoglycan size [191] is unchanged between the normal and diseased state. However, human cartilage metalloprotease activity is elevated by three- to ten-fold [192].

Severe osteoarthritis has been treated principally by corticosteroid administration. The production of hyaluronate in organ cultue by cells of the villous synovium is now known to be suppressed by both steroid suspensions and soluble hydrocortisone [193,194]. Whether this observation is clinically relevant is unknown. The inflamed joint does not necessarily show a decrease in hyaluronate concentration. However, large effusions would indicate an increase in the total hyaluronate production. Thus in some instances, a reduction in the hyaluronate synthetic activity is related to a reduction in effusion. Sodium hyaluronate injection has also been used in the treatment of acute osteoarthritis in horses [195–199], where it appears to improve lubrication of soft tissues, thus easing pain through decreased resistance to joint movement. Two human clinical trials have been performed using the intra-articular injection of hyaluronate as treat-

ment for symptomatic osteoarthritis [200,201]. Both studies concluded that, for most cases, hyaluronate was effective.

5.4. Rheumatoid arthritis and other diseases

Rheumatoid disease, unlike osteoarthritis, presents a consistent story of polysaccharide changes. Serum levels of circulating hyaluronate are elevated; 232 μg/l in the disease state; 42 μg/l in normals [202]. The pathological tissues produce more hyaluronate than normals and it is of a lower molecular mass [203–205]. Also, the protease activity of diseased synovial fluid is higher than normals. This latter point is significant since the breakdown of cartilage proteoglycan may contribute to the etiology of the disease [206–208].

Metabolic or genetic alterations in the content or composition of the GAGs are characteristic of other diseases such as systemic lupus erythematosus [209], pulmonary injury [210] and cirrhosis of the liver [211]. Additionally, hyaluronate and other GAGs have been investigated as potential dermal, corneal and burn wound treatments [212–215].

6. CONCLUSIONS

GAGs and proteoglycans in mammalian systems are ubiquitous. Compared to efforts in the fields of protein and nucleic acid research knowledge about mammalian polysaccharides is scant. Perhaps this disparity is related to the complexity of the extracellular matrix constituents. However, proteins and nucleic acids – two complex molecular classes – have yielded to investigative efforts following basic advances in separation technology and analytical methodology. Before theories of the structure and function relationships of GAGs and proteoglycans can be advanced, novel purification and detection systems must be developed. Such technology only now appears to be evolving. Additional basic research is needed.

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8. SUMMARY

This review of the mammalian extracellular matrix polysaccharides covered the glycosaminoglycans (GAGs) and their association into proteoglycans. As they necessarily pertain to the chromatographic and electrophoretic separations of these molecules, the structural features of the five principal GAGs were briefly reviewed. Much of the current structural work as well as the separation technology has been concerned with the sulfation state and copolymeric sequences of the individual classes of GAGs. The separation methods discussed included electrophoresis by agarose, acrylamide and cellulose acetate, high-performance liquid

chromatography (HPLC), ion-exchange, gel permeation and biospecific affinity methods. Since detection systems are an integral part of chemical separation technology, current thoughts about the best methods to assay GAGs or detect column fractions were discussed. These included polysaccharide-specific detection systems such as Alcian blue dye, 1,9-dimethylmethylene blue, bovine serum albumin–Coomassie blue, as well as non-specific carbohydrate detection systems such as the carbazole or indole hydrochloride methods. Instrumentation used in the detection of chromatography fractions for these molecules was discussed, since the usual ultraviolet detector, standard with HPLC equipment, is often unsatisfactory. The most sensitive specific detection method for GAGs is the use of monoclonal antibodies, which are only now becoming commercially available. The use of these antibodies, combined with HPLC separation, appears to be the best available biochemical technology for studying the extracellular matrix polysaccharides. Finally, the association between proteoglycans, GAGs and mammalian disease processes was reviewed, emphasizing mucopolysaccharidoses and arthritis. The early detection of both of these diseases is desired for effective counselling and treatment. Many of the methods discussed here have been applied, but others are yet to be tried in efforts to further that goal.

REFERENCES

1 L.A. Fransson, in G.O. Aspinall (Editor), Polysaccharides, Vol. 3, Academic Press, New York, 1985, pp. 337–415.
2 L.A. Fransson, L. Coster, I.A. Nieduszynski, C.F. Phelps and J.K. Sheenan, in S. Arnott, D.A. Rees and E.R. Morris (Editors), Molecular Biophysics of the Extracellular Matrix, Humana Press, Clifton, NJ, 1983, Ch. 5, pp. 95–118.
3 R.M. Mason, in Z. Deyl and M. Adam (Editors), Connective Tissue Research, Alan Liss, New York, 1981, pp. 87–112.
4 L. Sundblad, Acta Soc. Med, Ups., 58 (1955) 113–238.
5 J.E. Scott, in D. Glick (Editor), Methods of Biochemical Analysis, Vol. VIII, Interscience Publisher, New York, London, 1960, pp. 145–200.
6 J.E. Scott, in E.A. Balazs (Editor), Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 2, Academic Press, New York, 1970, pp. 1105–1119.
7 P.J. Roughley, Trans. Ophthalmol. Soc. U.K., 95 (1975) 369–371.
8 M.K. Cowman, E.A. Balazs, C.W. Bergmann and K. Meyer, Biochemistry, 20 (1981) 1379–1385.
9 M.B. Mathews and L. Decker, Biochim. Biophys. Acta, 498 (1977) 259–263.
10 B. Chakrabarti and E.A. Balazs, Biochim. Biophys. Res. Commun., 52 (1973) 1170–1176.
11 T.W. Barrett and J.E. Baxter, Physiol. Chem. Phys., 14 (1982) 19–29.
12 S. Arnott, A.K. Mitra and S. Raghunathan, J. Mol. Biol., 169 (1983) 861–872.
13 A. Davies, J. Gormally, E. Wyn-Jones, D.J. Wedlock and G.O. Phillips, Biochem. J., 213 (1983) 363–369.
14 J.K. Sheehan and E.D.T. Atkins, Int. J. Biomacromol., 5 (1983) 215–221.
15 A.K. Mitra, S. Raghunathan, J.K. Sheehan and S. Arnott, J. Mol. Biol., 169 (1983) 829–859.
16 A.K. Mitra, S. Arnott and J.K. Sheehan, J. Mol. Biol., 169 (1983) 813–827.
17 L. Roden, in W. Lennarz (Editor), The Biochemistry of Glycoproteins and Proteoglycans, Plenum Press, New York, 1980, Ch. 7, pp. 267–371.
18 M.E. Hammer and T.G. Burch, Invest. Ophthalmol. Visual Sci., 25 (1984) 1329–1332.
19 R.P. Millane, A.K. Mitra and S. Arnott, J. Mol. Biol., 169 (1983) 903–920.
20 S. Arnott and A.K. Mitra, in S. Arnott, D.A. Rees, and E.R. Morris (Editors), Molecular Biophysics of the Extracellular Matrix, Humana Press, Clifton, NJ, 1984, Ch. 3, pp. 41–67.
21 A.K. Mitra, S. Arnott, E.D. Atkins and D.H. Isaac, J. Mol. Biol., 169 (1983) 873–901.

22 D.A. Rees, E.R. Morris, J.F. Stoddardt and E.S. Stevens, Nature, 317 (1985) 480.
23 B. Casu, J. Choay, D.R. Ferro, G. Gatti, J.-C. Jacquinet, M. Ragazzi, P. Sinay and G. Torri, Nature, 322 (1986) 215-216.
24 M. Ragazzi, D.R. Ferro and A.J. Provasoli, J. Comput. Chem., 7 (1986) 105-112.
25 G.K. Hascall, J. Ultrastruct. Res., 70 (1980) 369-375.
26 R. Matalon and A. Dorfman, Proc. Natl. Acad. Sci., U.S.A., 60 (1968) 179-185.
27 D.C. Mitchell and T. Hardingham, Biochem. J., 202 (1982) 249-254.
28 M.X. Triscott and I. van de Rijn, J. Biol. Chem., 261 (1986) 6004-6009.
29 R.W. Mowry, J. Histochem. Cytochem., 8 (1960) 323-324.
30 J.E. Scott and J. Dorling, Histochemie, 5 (1965) 221-233.
31 D.J. Goldstein and R.W. Horobin, Histochem. J., 6 (1974) 157-174.
32 J. Tas, Histochem. J., 9 (1977) 205-230.
33 J.F. O'Brien and M.E. Emmerling, Anal. Biochem., 85 (1978) 377-386.
34 E.W. Gold, Anal. Biochem., 99 (1979) 183-188.
35 E.W. Gold, Biochim. Biophys. Acta, 673 (1981) 408-415.
36 P.M. Bartold and R.C. Page, Anal. Biochem., 150 (1985) 320-324.
37 R.L. Smith, E. Gilkerson, N. Kohaisu, T. Merchant and D.J. Schurman, Anal. Biochem., 103 (1980) 191-200.
38 W.P. Tew and R.N. Hotchkiss, J. Equine Vet. Sci., 1 (1981) 163-170.
39 R.E. Turner and M.K. Cowman, Arch. Biochem. Biophys., 237 (1985) 253-260.
40 K.B. Taylor and G.M. Jeffree, Histochem. J., 1 (1969) 199-204.
41 A.D. Sedgwick, A.R. Moore, A.Y. Al-Duaij, J.C. Edwards and D.A. Willoughby, Br. J. Exp. Pathol., 66 (1985) 445-453.
42 M. Petko, Stain Tech., 2 (1974) 65-67.
43 B. Szewczyk, Anal. Biochem., 130 (1983) 60-64.
44 J. Sandson, Arthritis Rheum., 11 (1968) 838-839.
45 D.N Barry and J.M. Bowness, Can. J. Biochem., 53 (1975) 713-720.
46 B.P. Toole and J. Gross, Dev. Biol., 25 (1971) 57-77.
47 U.B.G. Laurent and J.R.E. Fraser, Exp. Eye Res., 36 (1983) 493-504.
48 J.R.E. Fraser, T.C. Laurent, H. Pertoft and E. Baxter, Biochem. J., 200 (1981) 415-424.
49 C.J. Coulson and R. Girkin, Anal. Biochem., 65 (1975) 427-434.
50 S.S. Carlson, Anal. Biochem., 122 (1982) 364-367.
51 P.M. Sampson, R. Heimer and A.P. Fishman, Anal. Biochem., 151 (1985) 304-308.
52 U.B.G. Laurent and A. Tengblad, Anal. Biochem., 109 (1980) 386-394.
53 A.R. Poole, C. Webber, I. Pidoux, H. Choi and L.C. Rosenberg, J. Histochem. Cytochem., 34 (1986) 619-625.
54 B. Caterson, J.E. Christner and J.R. Baker, J. Biol. Chem., 258 (1983) 8848-8854.
55 Z. Dische, J. Biol. Chem., 167 (1947) 189-198.
56 T. Bitter and H.M. Muir, Anal. Biochem., 4 (1962) 330-334.
57 E.A. Balazs, K.O. Berntsen, J. Karossa and D.A. Swann, Anal. Biochem., 12 (1965) 547-558.
58 H.G. Rosenthal, J.P. Bentley and E.E. Albin, Connect. Tissue Res., 4 (1976) 155-161.
59 N. Jeansonne, B. Radhakrishnamurthy, E.R. Dalferes and G.S. Berenson, J. Chromatogr., 354 (1986) 524-529.
60 L.A. Elson and W.T.J. Morgan, Biochem. J., 27 (1933) 1824-1828.
61 D.A. Swann and E.A. Balazs, Biochim. Biophys. Acta, 130 (1966) 112-129.
62 Z. Dische and E. Borenfreund, J. Biol. Chem., 184 (1950) 517-522.
63 N. Ohno, I. Suzuki and T. Yadomae, Carbohydr. Res., 137 (1985) 239-243.
64 V.C. Hascall, R.L. Riolo, J. Hayward, Jr. and C.C. Reynolds, J. Biol. Chem., 247 (1972) 4521-4528.
65 G.W. Jourdian, M. Wolfman, R. Sarber and J. Distler, Anal. Biochem., 96 (1979) 474-480.
66 M. Koseki, A. Kimura and T. Tsurumi, J. Biochem., 83 (1978) 553-558.
67 M.A. Haney, Am. Lab., 17 (1985) 41-56.
68 M.A. Haney, Am. Lab., 17 (1985) 116-126.
69 T. Ohya and Y. Kaneko, Biochim. Biophys. Acta, 198 (1970) 607-609.
70 J.H. Ozegowski, D. Gerlach and W. Kohler, Zentralbl. Backteriol., 249 (1981) 310-322.

71 T. Yamagata, H. Saito, O. Habuchi and S. Suzuki, J. Biol. Chem., 243 (1968) 1523-1535.
72 M.O. Longas and R. Fleischmajer, Connect. Tissue Res., 13 (1985) 117-125.
73 J. Hollman, R. Niemann and E. Buddecke, Biol. Chem. Hoppe-Seyler, 367 (1986) 5-13.
74 O. Habuchi and N. Miyashita, Biochim. Biophys. Acta, 717 (1982) 414-421.
75 H. Inoue, K. Otsu, S. Suzuki and Y. Nakanishi, J. Biol. Chem., 261 (1986) 4470-4475.
76 R. DeGasperi, Y.T. Li and S.C. Li, J. Biol. Chem., 261 (1986) 5696-5698.
77 K. Nakazawa, N. Suzuki and S. Suzuki, J. Biol. Chem., 250 (1975) 905-911.
78 E.R. Ruter and H. Kresse, J. Biol. Chem., 259 (1984) 11771-11776.
79 R. Minami, S. Fujibayashi, C. Igarashi, Y. Ishikawa, K. Wagatsuma, T. Nakao and S. Tsugawa, Clin. Chim. Acta, 137 (1984) 179-187.
80 N. Ototani, M. Kikuchi and Z. Yosizawa, Carbohydr. Res., 88 (1981) 291-303.
81 M.Y. Tian, M. Yanagishita, V.C. Hascall and A.H. Reddi, Arch. Biochem. Biophys., 247 (1986) 221-232.
82 S. Suzuki, S. Suzuki, N. Nakamura and T. Koizuma, Biochim. Biophys. Acta, 428 (1976) 166-181.
83 I. Miyamoto and S. Nagase, J. Biochem., 95 (1984) 1331-1336.
84 C.J. Hutchison and R. Yasin, Dev. Biol., 115 (1986) 78-83.
85 G. Armand and M. Reyes, Biochem. Biophys. Res. Commun., 112 (1983) 168-175.
86 E.C. Lau and J.V. Ruch, Anal. Biochem., 130 (1983) 237-245.
87 H. Uchiyama, K. Okouchi and K. Nagasawa, Carbohydr. Res., 140 (1985) 239-249.
88 A.L. Fluharty, J.A. Glick, N. Matusewicz and H. Kihara, Biochem. Med., 27 (1982) 352-360.
89 G.J. Lee, D.W. Liu, J.W. Pav and H. Tieckelmann, J. Chromatogr., 212 (1981) 65-73.
90 C. Kodama, N. Ototani, M. Isemuar and Z. Yosizawa, J. Biochem., 96 (1984) 1283-1287.
91 K. Murata and Y. Yokoyama, Anal. Biochem., 146 (1985) 327-335.
92 K. Murata and Y. Yokoyama, Anal. Biochem., 149 (1985) 261-268.
93 I. Takazono and Y. Tanaka, J. Chromatogr., 288 (1984) 167-176.
94 H.G. Barth and D.A. Smith, J. Chromatogr., 206 (1981) 410-415.
95 H.G. Barth and F.E. Regnier, J. Chromatogr., 192 (1980) 275-293.
96 N.B. Beaty, W.P. Tew and R.J. Mello, Anal. Biochem., 147 (1985) 387-395.
97 P.J. Roughley and J.S. Mort, Anal. Biochem., 149 (1985) 136-141.
98 R.V. Iozzo, R. Marroquin and T.N. Wight, Anal. Biochem., 126 (1982) 190-199.
99 R.V. Iozzo and C.C. Clark, J. Biol. Chem., 261 (1986) 6658-6669.
100 N. Motohashi and I. Mori, J. Chromatogr., 299 (1984) 508-512.
101 L. Hronowski and T.P. Anastassiades, Anal. Biochem., 107 (1980) 393-405.
102 E.J. Schuchman and R.J. Desnick, Anal. Biochem., 117 (1981) 419-426.
103 A. Bertolotto and M.L. Magrassi, Electrophoresis, 5 (1984) 97-101.
104 K. Schmid, M. Wernli and R.B. Nimberg, Anal. Biochem., 121 (1982) 91-96.
105 L. Hronowski and T.P. Anastassiades, Anal. Biochem., 93 (1979) 60-72.
106 D.J. Newton, J.E. Scott and P. Whiteman, Anal. Biochem., 62 (1974) 268-273.
107 I.N. Hampson and J.T. Gallagher, Biochem. J., 221 (1984) 697-705.
108 M.K. Cowman, M.F. Slahetka, D.M. Hittner, J. Kim, M. Forino and G. Gadelrab, Biochem. J., 221 (1984) 707-716.
109 C.A. McDevitt and H. Muir, Anal. Biochem., 44 (1985) 612-622.
110 D. Heinegaard, Y. Sommarin and E. Hedbom, Anal. Biochem., 151 (1985) 41-48.
111 G.C. Dierckxsens, L. De Meyer and G.J. Tonino, Anal. Biochem., 130 (1983) 120-127.
112 J.R. Clamp, T. Bhatti and R.E. Chambers, in D. Glick (Editor), Methods in Biochemical Analysis. Vol. 19, Wiley, New York, 1971, pp. 229-344.
113 J.F. O'Brien, T. Gerritsen and A.C. Helmuth, Anal. Biochem., 56 (1973) 465-479.
114 J.P. Zanetta, W.C. Breckenridge and G. Vincendon, J. Chromatogr., 69 (1972) 291-304.
115 G. Bengtsson, T. Olivecrona, M. Hook, J. Riesenfeld and U. Lindahl, Biochem. J., 189 (1980) 625-633.
116 M.M. Klinger, R.U. Margolis and R.K. Margolis, J. Biol. Chem., 260 (1985) 4082-4090.
117 M. Del Rosso, R. Cappelletti, M. Viti, S. Vannucchi and V. Chiarugi, Biochem. J., 199 (1981) 699-704.
118 G. Lubec, Paediatr. Pathol. (Austria), 17 (1982) 591-596.

119 E.A. Turley and S. Roth, Nature, 233 (1980) 268-271.
120 A. Tengblad, Biochim. Biophys. Acta, 578 (1979) 281-289.
121 A. Tengblad, Biochem. J., 185 (1980) 101-105.
122 S. LeGledic, J.-P. Perin, F. Bonnet and P. Jolles, Biochem. Biophys. Res. Commun., 104 (1982) 1298-1305.
123 N.F. Boas, J. Biol. Chem., 181 (1949) 573-575.
124 H.E. Alburn and E.C. Williams, Ann. N.Y. Acad. Sci., 52 (1950) 971-976.
125 R.W. Jeanloz and E. Forchielli, J. Biol. Chem., 186 (1950) 495-511.
126 J.A. Cifonelli and M. Mayeda, Biochim. Biophys. Acta, 24 (1957) 397-400.
127 U.B. Laurent and K.A. Graneth, Exp. Eye Res., 36 (1983) 481-492.
128 R.L. Goldberg, J.D. Seidman, G. Chi-Rosso and B.P. Toole, J. Biol. Chem., 259 (1984) 9440-9446.
129 C.B. Underhill and B.P. Toole, J. Biol. Chem., 255 (1980) 4544-4949.
130 R.L. Goldberg, C.B. Underhill and B.P. Toole, Anal. Biochem., 125 (1982) 59-65.
131 C.B. Knudson and B.P. Toole, J. Cell Biol., 100 (1985) 1753-1758.
132 A. Engstrom-Laurent, U.B. Laurent, K. Lilja and T.C. Laurent, Scand. J. Clin. Lab. Invest., 45 (1985) 497-504.
133 L. Dahl, J.J. Hopwood, U.B. Laurent, K. Lilja and A. Tengblad, Biochem. Med., 30 (1983) 280-283.
134 U.B. Laurent and A. Tengblad, Anal. Biochem., 109 (1980) 386-394.
135 J.E. Christner, B. Caterson and J.R. Baker J. Biol. Chem., 255 (1980) 7102-7105.
136 B. Caterson, J.R. Baker, J.E. Christner and J.R. Couchman, J. Invest. Dermatol., 79 (1982) 45s-50s
137 H. Mehmet, P. Scudder, P.W. Tang, E.F. Hounsell, B. Caterson and T. Feizi, Eur. J. Biochem., 157 (1986) 385-391.
138 E.J. Thonar, M.E. Lenz, G.K. Klintworth, B. Caterson, L.M. Pachman, P. Glickman, R. Katz, J. Huff and K.E. Kuettner, Arthritis Rheum., 28 (1985) 1367-1376.
139 M. Hook, L. Kjellen, S.S. Johansson and J. Robinson, Ann. Rev. Biochem., 53 (1984) 847-869.
140 K.F. Greif and L.F. Reichardt, J. Neurosci., 2 (1982) 843-852.
141 K.F. Greif and A.S. Kelly, Brain Res., 275 (1983) 143-147.
142 W.D. Matthew and L.F. Reichardt, Prog. Brain Res., 58 (1983) 375-381.
143 K.M. Buckley, E.S. Schweitzer, G.P. Miljanich, L. Clift-O'Grady, P.D. Kushner, L.F. Reichardt and R.B. Kelly, Proc. Natl. Acad. Sci. U.S.A., 80 (1983) 7342-7346.
144 W.D. Matthew, R.J. Greenspan, A.D. Lander and L.F. Reichardt, J. Neurosci., 5 (1985) 1842-1850.
145 K.F. Greif and H.I. Trenchard, J. Cell Biochem., 26 (1984) 127-133.
146 C.F. Eldridge, J.R. Sanes, A.Y. Chiu, R.P. Bunge and C.J. Cornbrooks, J. Neurocytol., 15 (1986) 37-51.
147 K. Rostand, J.R. Baker, B. Caterson and J.E. Christner, J. Biol. Chem., 257 (1982) 703-707.
148 K. Nakazawa, D.A. Newsome, B. Nilsson, V.C. Hascall and J.R. Hassell, J. Biol. Chem., 258 (1983) 6051-6055.
149 B. Caterson, J.E. Christner, J.R. Baker and J.R. Couchman, Fed. Proc., Fed. Am. Soc. Exp. Biol., 44 (1985) 386-393.
150 D.C. Seldin, S. Nobuko, K.F. Austen and R.L. Stevens, Anal. Biochem., 141 (1984) 291-300.
151 D.C. Seldin, K.F. Austen and R.L. Stevens, J. Biol. Chem., 260 (1985) 11131-11139.
152 R.L. Stevens, K. Otsu and K.F. Austen, J. Biol. Chem., 260 (1985) 14194-14200.
153 J.W. Stevens, Y. Oike, C. Handley, V.C. Hascall, A. Hampton and B. Caterson, J. Cell Biochem., 26 (1984) 247-259.
154 S. Huber, M. van der Rest, P. Bruckner, E. Rodriquez, K.H. Winterhalter and L. Vaughan, J. Biol. Chem., 261 (1986) 5965-5968.
155 R.S. Fife, B. Caterson and S.L. Myers, J. Cell Biol., 100 (1985) 1050-1055.
156 B. Delpech and C. Halavent, J. Neurochem., 36 (1981) 855-859.
157 B. Delpech, C.R. Acad. Sci. (Paris), 290 (1980) 1067-1070.
158 H.U. Choi, L.H. Tang, T.L. Johnson and L. Rosenberg, J. Biol. Chem., 260 (1985) 13370-13376.

159 P.W. Gasper, M.A. Throll, D.A. Wenger, D.W. Macy, L. Ham, R.E. Dornsife, K. McBiles, S.L. Quackenbush, M.L. Kesel and E.L. Gillette, Nature, 312 (1984) 467-469.
160 P. Purkiss, D.A. Gibbs and R.W. Watts, Clin. Chim. Acta, 131 (1983) 109-121.
161 R. Matalon, R. Wappner, M. Deanching, I.K. Brandt and A. Horwitz, Ann. Clin. Lab. Sci., 12 (1982) 234-238.
162 V.A. McKusick. E.F. Neufeld and T.E. Kelley, in J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 4th ed., 1978, pp. 1281-1307.
163 E.R. Berman, J. Vered and G. Bach, Clin. Chem., 17 (1971) 886-890.
164 C.A. Pennock, J. Clin. Pathol., 22 (1969) 379-380.
165 K.C. Huang, K. Sukegawa and T. Orii, Clin. Chim. Acta, 151 (1985) 147-156.
166 R. Cappelletti, M.D. Posso and V.P. Chiarugi, Anal. Biochem., 99 (1979) 311-315.
167 J.J. Hopwood and J.R. Harrison, Anal. Biochem., 119 (1982) 120-127.
168 J.J. Hopwood and H. Elliott, Biochem. J., 229 (1985) 579-586.
169 G.J. Lee, J.E. Evans, H. Tieckelmann, J.T. Dulaney and E.W. Naylor, Clin. Chim. Acta, 104 (1980) 65-75.
170 G.J. Lee and H. Tieckelmann, J. Chromatogr., 222 (1981) 23-31.
171 C. Kodama, N. Ototani, M. Isemura, J. Aikawa and Z. Yosizawa, Clin. Chem., 32 (1986) 30-34.
172 K.S. Last, J.B. Stanbury and G. Embery, Arch. Oral Biol., 30 (1985) 275-281.
173 D.H. Fine and I.D. Mandall, J. Clin. Periodontol., 13 (1986) 533-546.
174 G. Embery and E. Whitehead, Calcif. Tissue Res., 22 (1976) 227-229.
175 G. Embery and H. Nordbo, Scand. J. Dent. Res., 87 (1979) 325-327.
176 G. Embery, W.M. Oliver and J.B. Stanbury, J. Periodont. Res., 14 (1979) 512-519.
177 G. Embery, W.M. Oliver, J.B. Stanbury and J.A. Purvis, Arch. Oral Biol., 27 (1982) 177-179.
178 L.S. Tipler and G. Embery, Arch. Oral Biol., 30 (1985) 391-396.
179 A. Linde, J. Dent. Res., 64 (1985) 523-529.
180 A. Hjerpe, C.A. Antonopoulos, B. Engfeldt and B. Wikstrom, Calcif. Tissue Int., 35 (1983) 496-501.
181 A. Hjerpe, C.A. Antonopoulos and B. Engfeldt, J. Chromatogr., 245 (1982) 365-368.
182 J.R. Couchman, B. Caterson, J.E. Christner and J.R. Baker, Nature, 307 (1984) 650-652.
183 K.S. Rostand, J.R. Baker, B. Caterson and J.E. Christner, Arthritis Rheum., 29 (1986) 95-105.
184 S.L. Carney, M.E. Billingham, H. Muir and J.D. Sandy, J. Orthop. Res., 3 (1985) 140-147.
185 K. Ida, Nippon Seikeigekagakkai Zasshi, 53 (1979) 949-962.
186 J. Schalkwijk, W.B. van den Berg, L.B. van de Putte and L.A. Joosten, Arthritis Rheum., 29 (1986) 532-538.
187 T.I. Morales and K.E. Kuettner, Biochim. Biophys. Acta, 705 (1982) 92-101.
188 C.J. Malemud, R.W. Moskowitz and V.M. Goldberg, Connect. Tissue Res., 12 (1984) 319-335.
189 A.J. Chuck, J. Murphy, J.B. Weiss and D.M. Grennan, Ann. Rheum. Dis., 45 (1986) 162-166.
190 I. Axelsson and A. Bjelle, Scand. J. Rheumatol., 8 (1979) 217-221.
191 E.R. Schwartz, C.R. Leveille, J.W. Stevens and W.H. Oh, Arthritis Rheum., 24 (1981) 1528-1539.
192 J. Martel-Pelletier, J.P. Pelletier, J.M. Cloutier, D.S. Howell, L. Ghandur-Mnaymneh and J.F. Woessner, Jr., Arthritis Rheum., 27 (1984) 305-312.
193 S.L. Myers, Arthritis Rheum., 28 (1985) 1275-1282.
194 S.L. Myers and T.A. Christine, Arthritis Rheum., 26 (1983) 764-770.
195 N. Rydell and E.A. Balazs, Clin. Orthop., 80 (1971) 25-32.
196 D.J. Nizolek and K.K. White, Cornell Vet., 71 (1981) 355-375.
197 D.H. Irwin, J.S. Afr. Vet. Assoc., 51 (1980) 231-233.
198 B.J. Hilbert, G. Rowley, K.N. Antonas, C.A. McGill, J.A. Reynoldson and C.D. Hawkins, Aust. Vet. J., 62 (1985) 182-184.
199 C.W. McIlwraith, J. Am. Vet. Med. Assoc., 180 (1982) 239-250.
200 O. Namiki, H. Toyoshima and N. Morisaki, Int. J. Clin. Pharmacol. Ther. Toxicol., 20 (1982) 501-507.
201 S. Kopp, B. Wenneberg, T. Haraldson and G.E. Carlsson, J. Oral Maxillofac. Surg., 43 (1985) 429-435.

202 A. Engstrom-Laurent and R. Hallgreen, Ann. Rheum. Dis., 44 (1985) 83-88.
203 I.M. Dahl and G. Husby, Ann. Rheum. Dis., 44 (1985) 647-657.
204 L.B. Dahl, I.M. Dahl, A. Engstrom-Laurent and K. Granath, Ann. Rheum. Dis., 44 (1985) 817-822.
205 E. Vuorio, S. Einola, S. Hakkarainen and R. Penttinen, Rheumatol. Int., 2 (1982) 97-102.
206 J.J. Steinberg, S. Tsukamato and C.B. Sledge, Biochim. Biohys. Acta, 757 (1983) 47-58.
207 K. Shimizu, K. Susuda, M. Minami and N. Katsura, Nippon Seikeigekagakkai Zasshi, 55 (1981) 1585-1594.
208 K. Shimizu, K. Susuda, M. Minami and N. Katsura, Nippon Seikeigekagakkai Zasshi, 55 (1981) 1595-1605.
209 P. Faaber, T.P. Rijke, L.B. van de Putte, P.J. Capel and J.H. Berden, J. Clin. Invest., 77 (1986) 1824-1830.
210 S.C. Sahu and A.G. Ulsamer, Toxicol. Lett., 5 (1980) 283-286.
211 A. Engstrom-Laurent, L. Loof, A. Nyberg and T. Schroder, Hepatology, 5 (1985) 638-642.
212 G. Abatangelo, M. Martelli and P. Vecchia, J. Surg. Res., 35 (1983) 410-416.
213 C.J. Doillon and F.H. Silver, Biomaterials, 7 (1986) 3-8.
214 G. Arzeno and D. Miller, Arch. Ophthalmol., 100 (1982) 152.
215 D. Saric and M. Reim, Fortschr. Ophthalmol., 81 (1984) 588-591.