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PROTEOGLYCANS, GLYCOSAMINOGLYCANS AND SULFATED POLYSACCHARIDES FROM CONNECTIVE TISSUES

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THE GLYCOSAMINOGLYCAN COMPONENTS OF PROTEOGLYCANS

Glycosaminoglycans are a family of heterogeneous polysaccharides, composed of repeating disaccharides consisting of a hexosamine (usually *D*-glucosamine or *D*-galactosamine) glycosidically linked to either hexuronic (*D*-glucuronic or *L*-iduronic) acid or *D*-galactose. There are four general classes of glycosaminoglycans defined by the chemical structure of their repeating disaccharide units: 1) chondroitin sulfate and its modified form dermatan sulfate; 2) hyaluronic acid; 3) keratan sulfate; and 4) heparan sulfate and heparin.

Chondroitin sulfate is composed of repeating disaccharide units of $\beta-D-$glucuronic acid $1 \rightarrow 3$ linked glycosidically to N-acetyl $\beta-D-$galactosamine (a, Fig. 1). The N-acetyl $D-$galactosamine units carry a sulfate ester in either position 4 or 6, and, accordingly, the polysaccharide is designated as chondroitin 4-sulfate or chondroitin-6 sulfate.* Only rarely are all the hexosamine residues sulfated at the same position, and a hybrid structure with both 4- and 6-sulfate groups present in the same molecule (but on separate *D*-galactosamine residues) is the rule rather than the exception (Senno et al., 1975; Michelacci & Dietrich, 1976; Faltynek & Silbert, 1978; Michelacci et al., 1979).

Apart from the structural heterogeneity indicated above, the chondroitin sulfate chains may vary in average length from one tissue to another and within the same tissue. Usually, the average molecular weight of the chondroitin sulfate chains decreases from the young to the adult articular cartilage (Mathews & Glacov, 1966).

The chains of chondroitin sulfate (and also of other glycosaminoglycans) are linked to protein through the typical tetrasaccharide glucuronosyl-galactosyl-galactosyl-xylose (Lindahl & Rodén, 1972), as shown in b, Fig. 1.

Dermatan sulfate was first isolated from pig skin. *D*-galactosamine was immediately recognized as the amino sugar of dermatan sulfate, whereas the identity of the hexuronic acid remained unknown for many years. Structural analyses identified α-*L*-iduronic acid** in this glycosaminoglycan (Hoffman et al., 1956), and subsequently studies established that the positions of the glycosidic linkages are analogous to those observed for the chondroitin sulfate and also determined that the sulfate ester is located mainly at carbon 4 of the *D*-galactosamine residues (Cifonelli et al., 1958; Mathews, 1958) (c, Fig. 1).

Small amounts of *D*-glucuronic acid is present in dermatan sulfate, even after extensive fractionation, and it was conclusively demonstrated that this hexuronic acid is an integral part of the dermatan sulfate molecule (Fransson & Rodén, 1967a, b). Thus, dermatan sulfate may be viewed as a copolymer of disaccharide units containing both *L*-iduronic and *D*-glucuronic acid.

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*Studies about the distribution of chondroitin 4- and 6-sulfate in articular and endochondral ossification cartilages showed that the concentration of the 4-sulfate disaccharide units decreases sharply from the ossification cartilage to the articular surface, whereas that of 6- sulfated units does not change (Mourão, 1988; Harab & Mourão, 1989). In cartilage from the articular surface of the epiphysis in adults, where the ossification process is complete, the concentration of 4-sulfated units in the chondroitin chains is also low (Mourão et al., 1976; Mourão, 1988; Harab & Mourão, 1989). These observations suggest that chondroitin 4-sulfate is important in the ossification process, whereas chondroitin 6-sulfate is related to the structural integrity of the cartilage.

**It should be noted that the α-linkage of *L*-iduronic acid is analogous to the β-glucuronidic linkages of the chondroitin sulfate.

The repeating disaccharide unit of hyaluronic acid (d, Fig. 1) is similar to that of chondroitin sulfate, differing only in that the amino sugar is *D*-glucosamine rather than *D*-galactosamine, while the hexuronic acid is *D*-glucuronic acid in both, and the positions and anomeric configurations of the glycosidic linkages are the same. Hyaluronic acid however has no sulfate groups and apparently is not covalently linked to protein.

N-acetyllactosamine, a commonly occuring disaccharide component in glycoprotein, is the repeating unit of keratan sulfate (e, Fig. 1). Sulfate groups are present at carbon 6 of the N-acetyl *D*-glucosamine units, and in minor amounts on some of the *D*-galactose residues.

The chemistry of heparin and heparan sulfate has been the subject of several studies (Silva & Dietrich, 1975; Lindahl & Höök, 1978; Rodén & Horowitz, 1978; Rodén, 1980; Dietrich et al., 1983; Nader et al., 1987). The repeating disaccharide unit of both polymers is composed of *D*-glucosamine and hexuronic acid residues, although both glycosaminoglycans show a high structural heterogeneity. Thus, some amino sugars may carry an N-sulfate group, while other glucosamine residues are N-acetylated. The glucosamine residues may also carry an ester sulfate at the OH-6 position, and, in addition, the hexuronic (*L*-iduronic) acid residue may be sulfated at carbon 2.

GENERAL FEATURES OF PROTEOGLYCAN STRUCTURE

Proteoglycans are proteins that contain one or more covalently bound glycosaminoglycan chains. These molecules have a high charge density, due to the sulfate esters and carboxyl groups of the glycosaminoglycan chains, which leads to a large osmotic swelling pressure (Comper & Laurent, 1978; Maroudas, 1979). In the cartilage matrix, the concentration of proteoglycan can exceed 10% of the wet weight and occupies a volume of no more than 10 ml/g (dry weight) (Torchia et al., 1977). In free solution the proteoglycans expand to occupy some five times the volume they have in the tissue. Thus, in the matrix the high concentration of proteoglycans exerts a swelling pressure on the inextensible collagen fibrilar meshwork, which gives cartilages their resiliency, their ability to withstand compressive load with minimal deformation and provides a low-friction surface in a joint (Comper & Laurent, 1978).

The compressive properties of cartilage correlate most closely with the charge density of the anionic groups on the glycosaminoglycan chains and hence with the proteoglycan concentration (Maroudas et al., 1986). Thus, the higher the charge density, the less tissue deformation for a given compressive load. An interesting biological example of this can be seen in the reduced size of the cartilage

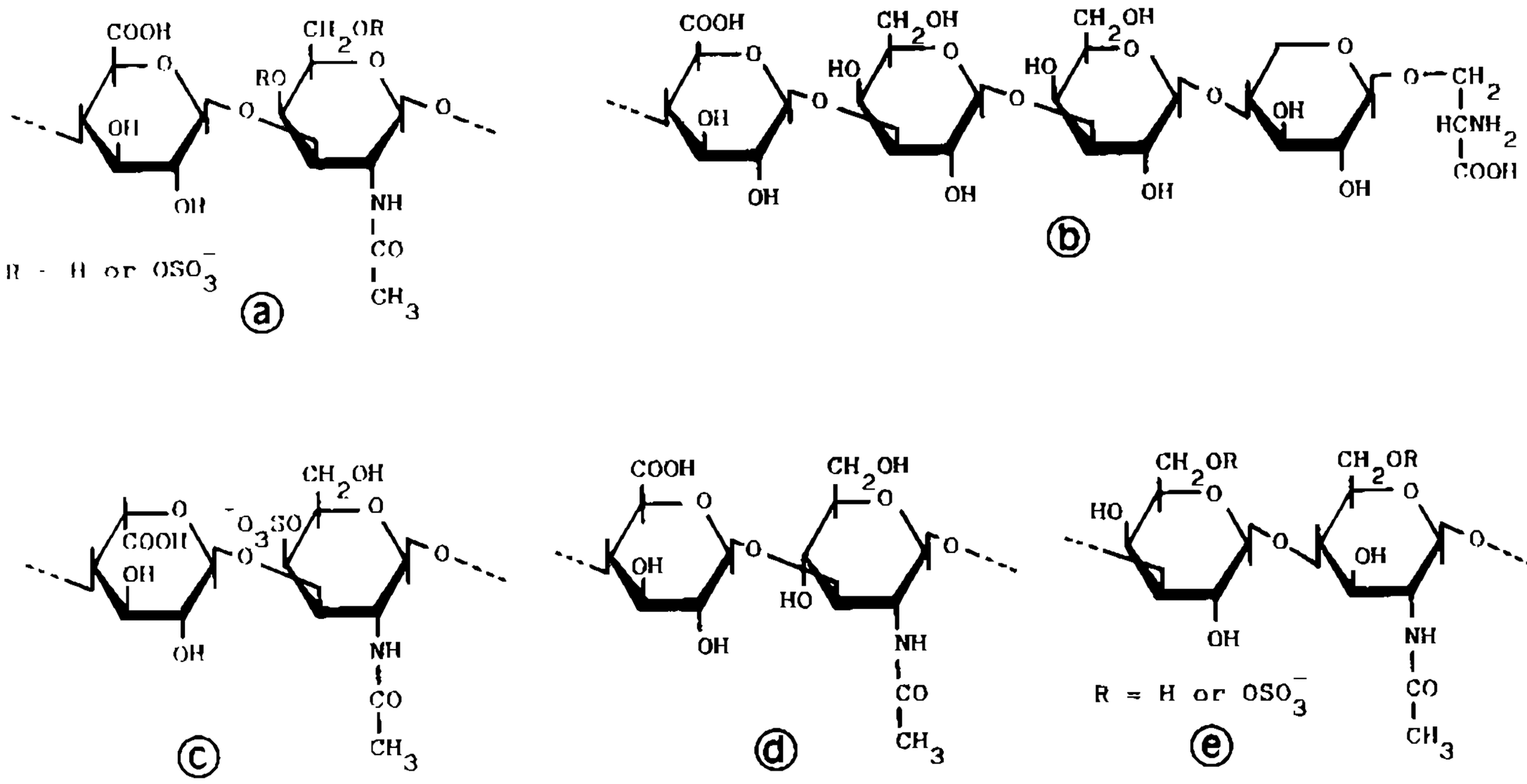

Fig. 1: chemical structure of the dominant disaccharide units of chondroitin 4/6-sulfate (a), dermatan sulfate (c), hyaluronic acid (d) and keratan sulfate (e). b shows the linkage region of chondroitin sulfate to protein.

growth plates in the brachymorphic mouse, where the proteoglycan is biosynthetically undersulfated (Orkin et al., 1977). Another heritable disorder due to defective sulfation of chondroitin sulfate was described in patients with spondylepiphyseal dysplasia, where the molecule is also undersulfated (Mourão et al., 1973; Mourão et al., 1981). The articular cartilages of these patients, unlike those of normal subjects, are unable to absorb compressive loads, and the ensuing higher tensions transmitted to the bones induce a process of bone remodeling and resorption, besides irregularities in the articular surface (Toledo et al., 1978).

Over the past two decades, there has been a tremendous upsurge in the study of connective tissue proteoglycans, largely as a result of the development of new techniques for the isolation of these compounds with high yields and in undegraded form. A major step occurred when Hascall & Sadjera (1969) discovered that denaturing solvents extract proteoglycan with high yields from cartilage. This solvent reversibly dissociate proteoglycan aggregates in the extracellular matrix and allow the proteoglycan monomers to be solubilized. Once solubilized, the proteoglycans can be purified by taking advantage of the unique properties of their glycosaminoglycan chains, which give the parent proteoglycans high anionic charge, high buoyant density and large hydrodynamic domains relative to globular proteins. Thus, ion exchange chromatography, isopycnic CsCl density gradient centrifugation and molecular sieve chromatography have all been used alone or in combination to purify proteoglycans. For cartilage proteoglycans, isopycnic CsCl gradients have proved particularly useful for the elucidation of the proteoglycan aggregate structure (Hascall, 1988).

The basic experimental approach for cartilage involve the extraction of the proteoglycans with denaturing or "dissociative" solvent (mainly 4 M

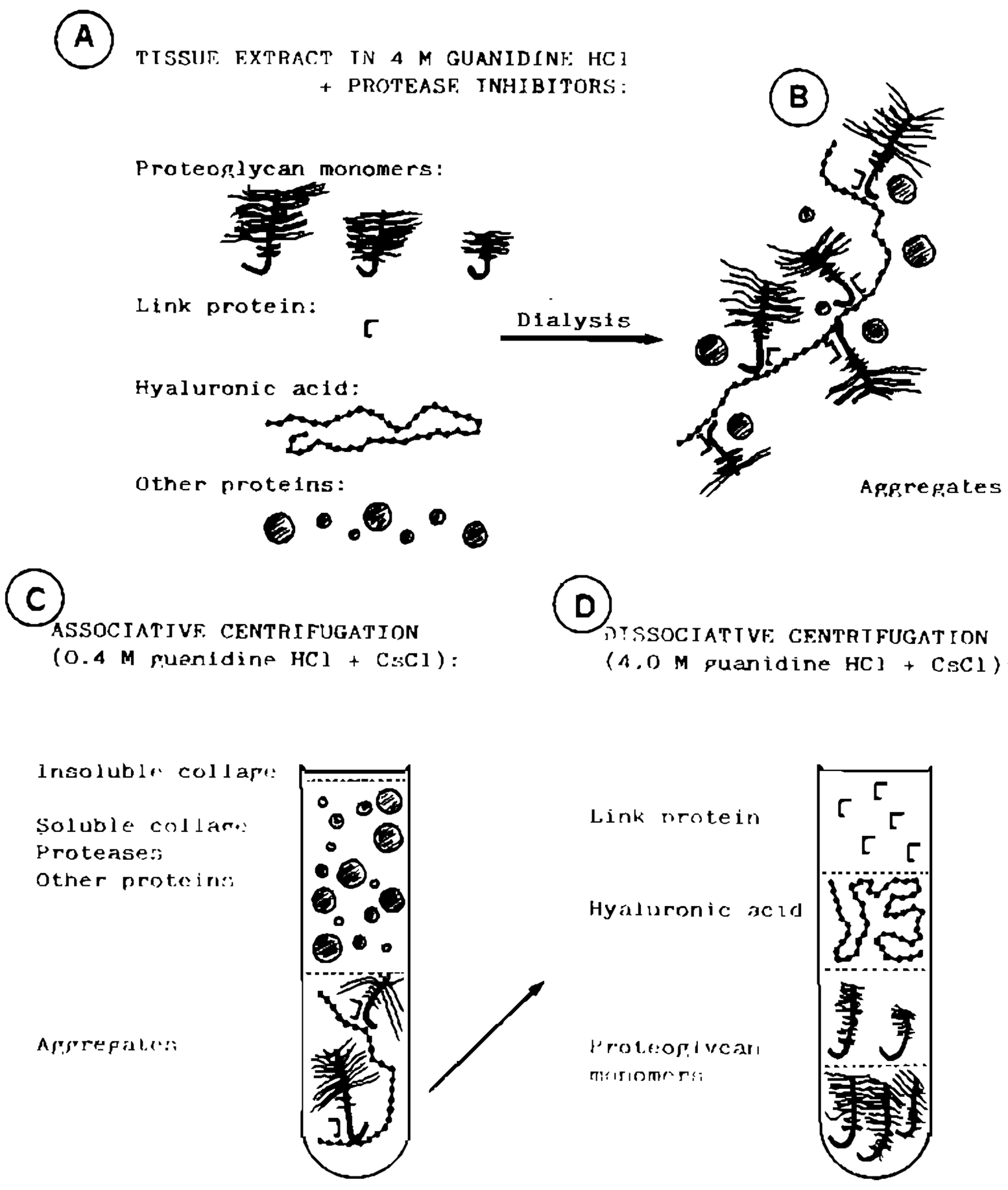

Fig. 2: diagrammatic representation for isolation of proteoglycan aggregates by extraction with guanidine HCl and separation of their components (monomers, link protein, and hyaluronic acid) by density-gradient centrifugation. Modified from Rodén (1980).

guanidine HCl) in the presence of protease inhibitors. If the extract is maintained in a dissociating medium and subjected to density-gradient centrifugation in CsCl, proteoglycan monomers are separated from other components of the extract and are found in the highest-density fraction which sediments to the bottom of the centrifuge tube (see Fig. 2).

The dissociation of the native proteoglycan aggregates is a reversible process, and combination of the isolated monomers with the material at the top of the gradient yields aggregates detectable by analytical ultracentrifugation. Such aggregate formation requires reduction of the concentration of the dissociating agent. In an alternative experimental approach, the initial tissue extract may be dialyzed to reduce the guanidine HCl concentration to 0.4 M, and density-gradient centrifugation of this material then yields the proteoglycan in the form of aggregates (see Fig. 2). Subsequent centrifugation of the aggregates under dissociative conditions permit the isolation of proteoglycan monomers separated from other specific components of the aggregates.

One such component is hyaluronic acid, which interacts specifically with the proteoglycan to form large aggregates (Hardingham & Muir, 1972). Also of major importance in the formation of native aggregates is the participation of "link protein" (Hascall & Sadjera, 1969), which apparently stabilizes the interaction between hyaluronic acid and proteoglycans.

An increasing number of extracellular proteoglycans have been purified and characterized in the last few years. The best studied is the large aggregating proteoglycan extracted from cartilage (for a review see Heinegard & Oldberg, 1989). This molecule has a protein core of Mr = 210,000 (Doege et al., 1988) to which many different types of glycosaminoglycan chains are covalently bound. Several distinct domains can be identified in the core protein. In the amino-terminal part, a globular domain (G-1, Fig. 3), containing a double and a single loop, forms a structure that allows specific interaction with a high molecular weight hyaluronic acid. The binding requires five repeat disaccharide units of hyaluronic acid. Therefore, many proteoglycan molecules can bind to a single hyaluronic acid molecule. The binding is stabilized by the link protein, which binds to the hyaluronic acid as well as to the hyaluronic acid binding region of the proteoglycan molecule. The link protein shows major homology with the hyaluronic acid binding region of the proteoglycan core protein, and both bind to hyaluronic acid with the same specificity and with similar strength.

The amino-terminal hyaluronic acid binding region of the cartilage proteoglycan is separated from a second globular domain (G-2, Fig. 3) by a short extended peptide stretch. Beyond the G-2 are the keratan sulfate-rich and the chondroitin sulfate-rich region. This latter region contains high amounts of Ser-Gly units, representing the putative signal for the transferase involved in the syn-

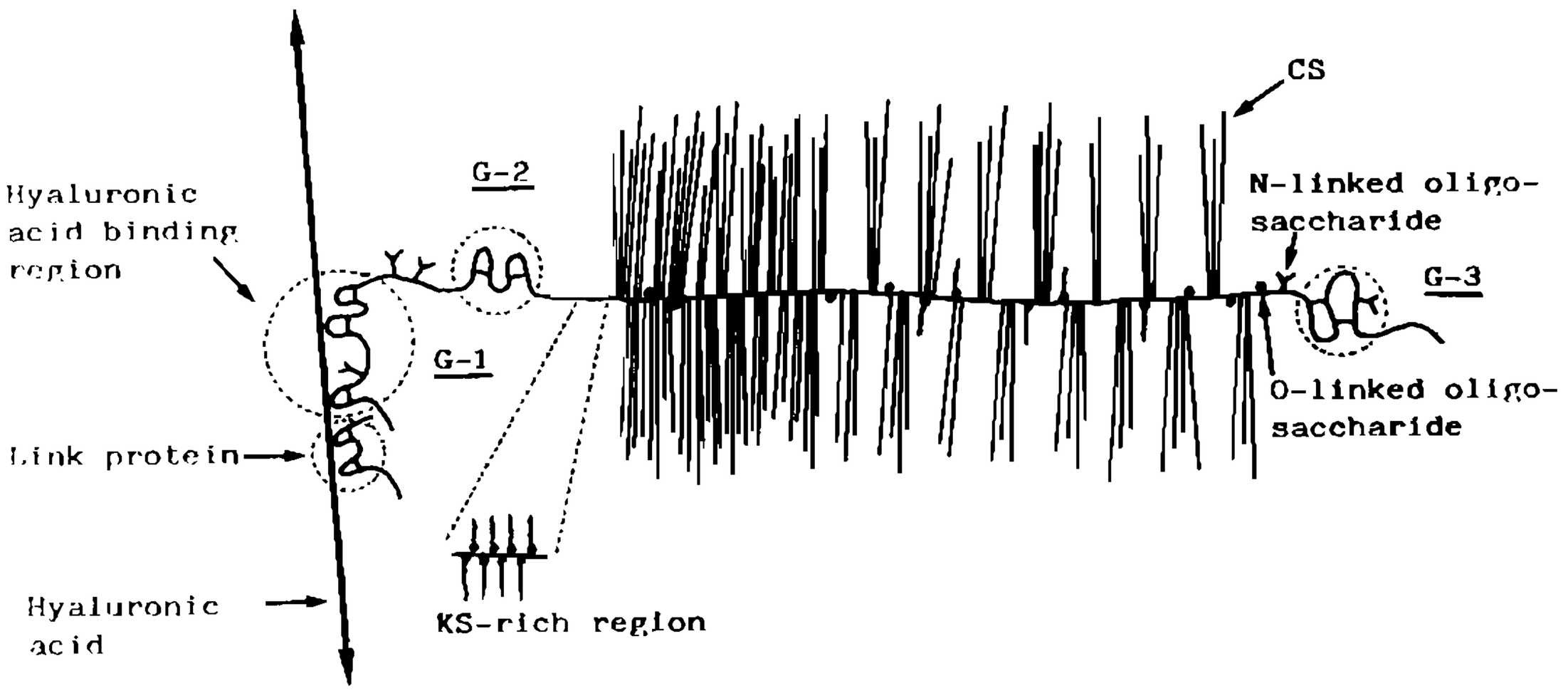

Fig. 3: schematic presentation of the large aggregating proteoglycan extracted from cartilage. The central protein core with three globular domains (G1, G2 and G3) is substituted with a large number of chondroitin sulfate chains (CS), keratan sulfate (KS), and oligosaccharides. See text for other details. Modified from Heinegard & Oldberg (1989).

thesis of the chondroitin sulfate chains. Almost all of the Ser-Gly structures are substituted by chondroitin sulfate chains, and therefore the number of these chains in the proteoglycan closely reflects the number of Ser-Gly structures.

The proteoglycan domain closest to the carboxyl-terminal is the third globule (G-3, Fig. 3). This structure has a homology with a hepatic lectin.

The major feature of this large proteoglycan is its great number of negatively charged glycosaminoglycan side chains: about 100 negatively charged carboxyl and sulfate groups (Heinegard & Oldberg, 1989). Thus this proteoglycan provide a domain with a high density of fixed, negatively charged groups.

Cartilages have two other types of proteoglycans, referred to as small proteoglycans, that, in contrast to the large proteoglycan, carry only one or two glycosaminoglycan chains. The side chains are either chondroitin sulfate or dermatan sulfate, varying with the tissue of origin (Heinegard & Sommarin, 1987; Sampaio et al., 1988).

OTHER SULFATED POLYSACCHARIDES FROM CONNECTIVE TISSUES

During the last years we have searched for sulfated polysaccharides in different invertebrate connective tissues. The main purpose of such studies is to compare these polysaccharides with the well known proteoglycans (and glycosaminoglycans) that occur in vertebrate tissues and to relate their structure with physicochemical and biological properties. In order to undertake these studies we look for tissues in invertebrates with properties similar to those of vertebrate connective tissues. Among these properties are the structural function; the relatively high proportion of extracellular matrix and relatively low proportion of cells; and the high concentration of proteoglycan or other acidic polysaccharide. We concentrated our studies on the tunic of ascidians (Chordata-Tunicata) and the body wall of the sea cucumber (Echinodermata-Holothuroidea).

Sulfated polysaccharides from the tunic of ascidians: the tunic of ascidians is an external supportive and protective skeleton (Barnes, 1980). It consists of dense bundles of fibrillar material embedded in a loose network of fine fibrils (unpublished observations), an organization reminiscent of the vertebrate cartilage.

High amounts of sulfated polysaccharides were extracted from the ascidian tunic (Albano & Mourão, 1983) and separated into several fractions by a combination of ion exchange and gel filtration chromatography (Albano & Mourão, 1986). The major fraction is a high molecular weight sulfated *L*-galactan (average Mr above 10^6), whose structure was determined using a vast array of techniques. The nature of their glycosidic linkages and the position of sulfation were determined by methylation. They were confirmed by periodate oxidation and nuclear magnetic resonance spectroscopy (Mourão & Perlin, 1987; Pavão et al. 1989a, b).

Figure 4 summarizes the main *L*-galactose units found in the ascidian sulfated *L*-galactan, and the major reactions employed to determine these structures (Albano et al., 1990). The methylation studies of this sulfated *L*-galactan indicate that the polymer is constituted mainly of a carbohydrate core of *L*-galactose linked glycosidically through position 1 → 4 and sulfated at position 3 (1a, Fig. 4). Therefore, 2, 3, 6-tri-*O*-methyl *L*-galactose is the main methyl ether derivative obtained from desulfated *L*-galactan (4, Fig. 4), whereas 2, 6-di-*O*-methyl *L*-galactose is the predominant methyl ether derivative obtained from sulfated *L*-galactan (2, Fig. 4). In addition, 6-*O*-methyl derivative of *L*-galactose is obtained in equimolar proportion with the 2, 3, 4, 6-tetra-*O*-methyl derivative through methylation of intact *L*-galactan (1, Fig. 4). This indicate that some of the 3-sulfated and 1 → 4-linked units of the central polysaccharide core are substituted at 0-2 position by non-sulfated *L*-galactopyranose units (1b, Fig. 4). Finally, the formation of 2, 3, 6-tri-*O*-methyl *L*-galactose from intact *L*-galactan indicates that some of the 1 → 4-linked units are not sulfated (1c, Fig. 4).

The structure of the sulfated *L*-galactan from ascidians is unique among previously described polysaccharides. The main galactose-rich sulfated polysaccharides described in living tissues are keratan sulfate and carrageenans. Keratan sulfate, which occurs mainly in mammalian cartilages and cornea, is composed of β-*D*-galactopyranose units 1 → 4-glycosidically linked to N-acetyl-*D*-glucosamine 6-sulfate (e, Fig. 1). The algal carrageenans present a more heterogeneous structure. They have a linear chain of β-*D*-galactopyranose residues linked glycosidically through position 1 → 3 to α-galactopyranose. The α-galactopyranose can occur in *D* or *L* form, or can be partly con-

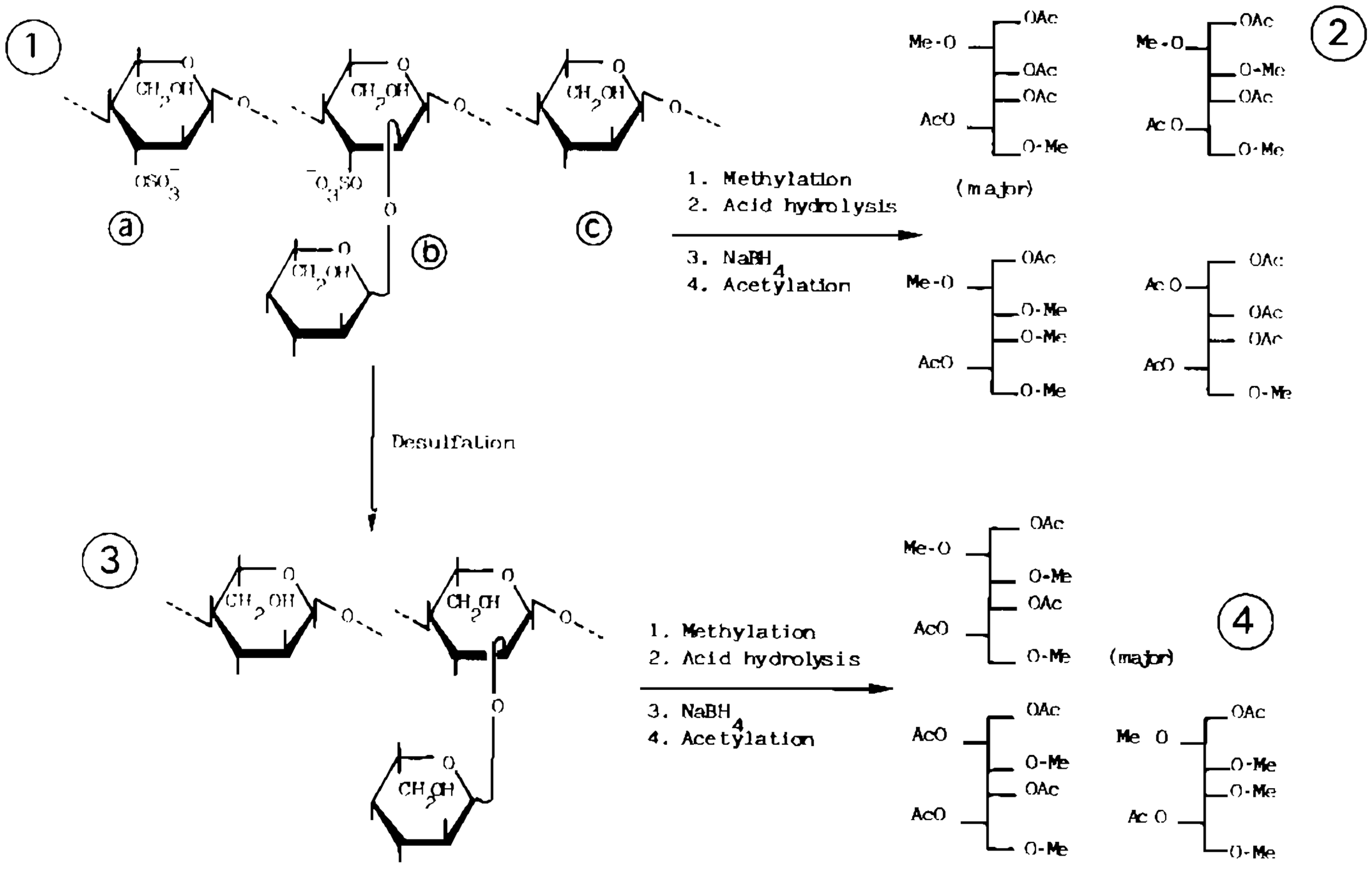

Fig. 4: structures of the main *L*-galactose units in the sulfated *L*-galactan from ascidian, and the major reactions employed to determine these structure. See text for other details.

verted to 3, 6-anhydro forms. The sulfate ester may occur at position 2, 4 or 6 of the galactose residues (Painter, 1983).

The ascidian *L*-galactan differs from these previously described sulfated polysaccharides not only in the type of linkage and position of sulfation, but also in the presence of branching, while both keratan sulfate and carrageenans are linear polysaccharides. Furthermore, the ascidian glycans are the first polysaccharides that contain large amounts of *L*-galactose and not its *D*-isomer.

A marked structural variation was observed among sulfated *L*-galactan from different species of ascidians. This polysaccharide from the ascidian *Clavelina* sp. is distinguished by its low content of nonreducing end units (Pavão et al., 1989b; Pavão et al., 1990) while the sulfated *L*-galactan from *A. nigra* has low sulfate content and high amounts of nonreducing end residues (Pavão et al., 1989b).

Finally, in order to compare the ascidian-sulfated glycans with proteoglycans extracted from cartilage, the ascidian tunic was submitted to extraction with guanidine HCl. The sulfated polysaccharides of low molecular weight could be extracted with this solvent. However, most of the sulfated *L*-galactan remained in the tunic (Albano & Mourão, 1986). Possibly, this polysaccharide is more resistant to guanidine HCl extraction than cartilage proteoglycans because of its high molecular weight. Therefore, it remains to be clarified whether the ascidian *L*-galactan is linked to protein.

Sulfated polysaccharide from the body wall of sea cucumber: the body wall of the sea cucumber is formed mainly by collagen fibers embedded in an amorphous matrix. Small irregular microfibrils, which resemble the proteoglycans of mammalian connective tissue, form bridges between the collagen fibers (Junqueira et al., 1980).

The sulfated polysaccharides from the body wall of the sea cucumber can be separated into various fractions that differ markedly in molecular weight and chemical composition (Mourão & Bastos, 1987; Vieira & Mourão, 1988). One fraction is primarily a sulfated α-*L*-fucan. Another fraction, which represents the major portion of the sea cucumber-sulfated polysaccharides, has an unusual structure composed of a chondroitin sulfate-like core, containing side chain disaccharide units of sulfated fucopyranosyl linked to approximately half of the glucuronic acid moieties through the 0-3 position of the acid (Vieira & Mourão, 1988).

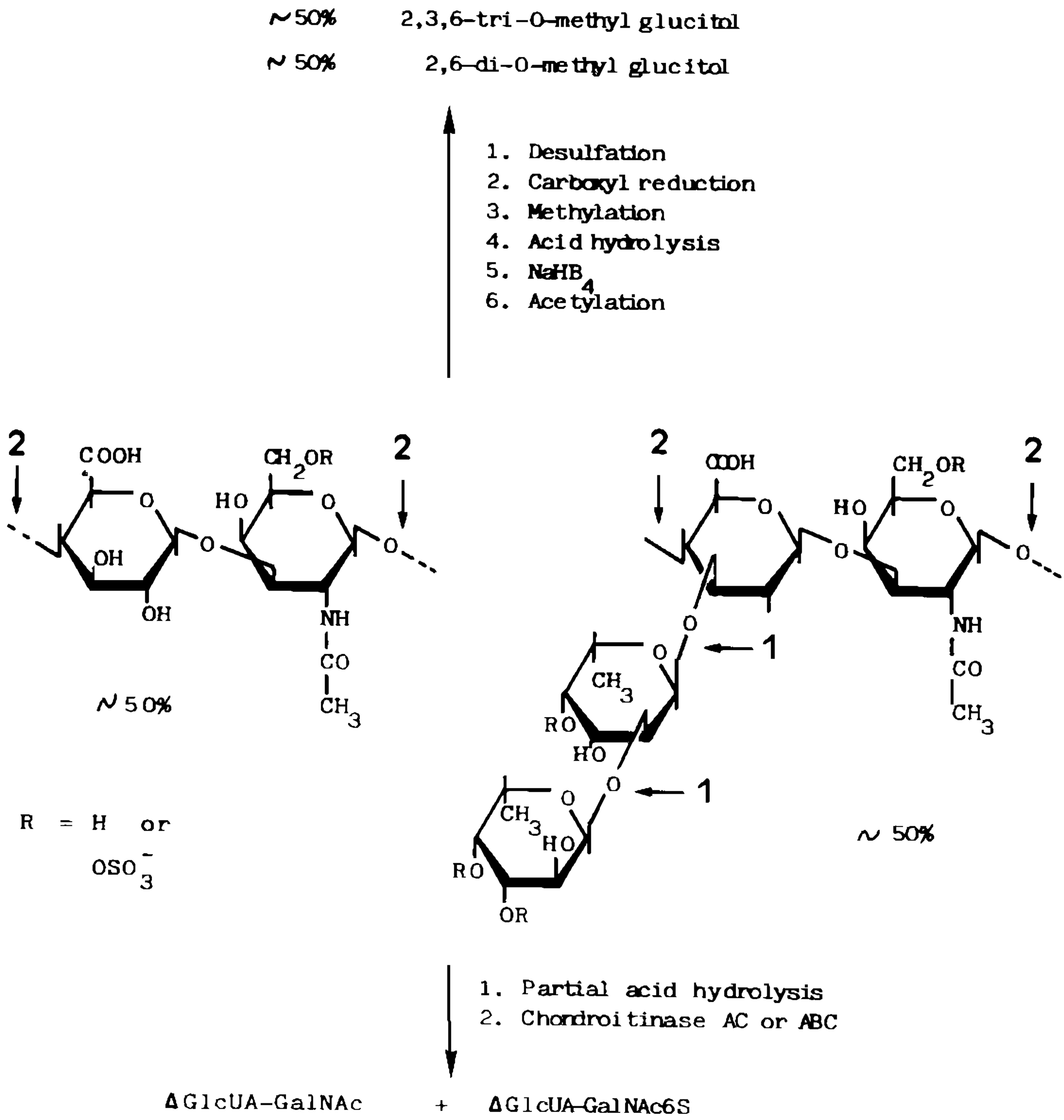

Fig. 5: structure of the main disaccharide units of the fucose-branched chondroitin sulfate from the sea cucumber, and the major reactions employed to determine these structures. See text for other details.

Figure 5 shows the hypothetical structure for the major components of the fucose-branched chondroitin sulfate. The presence of these unusual fucose branches obstruct the access of chondroitinases to the chondroitin sulfate core. However, after partial acid hydrolysis (1, Fig. 5), which removes the sulfated fucose residues, the polymer is degraded by chondroitinases (2, Fig. 5) into 6-sulfated and nonsulfated disaccharides (Vieira & Mourão, 1988).

Methylation of the fucose-branched chondroitin sulfate, after desulfation and carboxyl-reduction, forms approximately equimolar proportions of 2, 3, 6-tri-*O*-methyl and 2, 6-di-*O*-methyl-glucitol (Fig. 5). This observation indicates that half of the *D*-glucuronic acid residues are fucosylated.

More recently, the sulfated polysaccharides were extracted from the body wall of sea cucumber with guanidine HCl, in the presence of protease inhibitors. The molecular weight of the fucose-branched chondroitin sulfate, extracted by guanidine HCl, changes dramatically after β-elimination, indicating that this polysaccharide is O-linked to a polypeptide core (unpublished observations).

Major conclusions: our data on the sulfated polysaccharides from invertebrate connective tissues, such as the sulfated *L*-galactans from ascidians and the fucose-rich sulfated polysaccharides from the sea cucumber, are of considerable interest, as they show unusual examples of variants of polyanionic glycans with structural function in living tissues. From our results it is possible to speculate that such biological function in animal tissues may be served by sulfated polymers showing considerable variations as observed among the proteoglycans (and glycosaminoglycans) from vertebrate connective tissues and these unusual sulfated polysaccharides from invertebrates.

Interestingly, all these polymers possess extensive branching and abundance of sulfate ester, which may increase their water binding capacity and therefore leads to a large osmotic swelling pressure in the extracellular matrix. In addition, they have a very high molecular weight (usually above 10^6 daltons). However, in the sulfated *L*-galactan from ascidian the principal core component is a polysaccharide rather than a polypeptide as in the vertebrate and sea cucumber proteoglycans.

Sulfated polysaccharides are widespread in nature, occurring in great concentrations in marine algae and in the connective tissues of vertebrates. The sulfated polysaccharides described in the tunic of ascidian and in the body wall of the sea cucumber appear to be "intermediate" in nature with respect to these two other groups of sulfated polysaccharides. For example, the fucose-branched chondroitin sulfate resembles the animal glycosaminoglycans. On the other hand, the sulfated fucan from the same invertebrate reminds us of the marine algae fucoidan, since both polysaccharides are made up of sulfated α-L-fucopyranosyl units. Similarly, the sulfated *L*-galactan from the ascidian tunic, which is composed maily of $\alpha-L-$galactopyranose residues, resembles the marine algae carrageenan. Moreover, these invertebrate polysaccharides are unique among known sulfated polysaccharides in living tissues, which raises interesting questions concerning their metabolism and biological activities.

The biological relevance of these unusual polysaccharides found in marine invertebrates is still unclear. Possibly, the presence of *L*-isomers of galactose in the ascidian polysaccharides and of fucose branches in the chondroitin sulfate from the sea cucumber make these polysaccharides resistant to degradation by *D*-galactosidases, hyaluronidases and chondroitinases, and therefore prevents the digestion of the ascidian tunic and of the sea cucumber body wall by microorganisms present in the marine environment.

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