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**AORTIC GLYCOSAMINOGLYCANS IN ATHEROSCLEROSIS- SUSCEPTIBLE
AND -RESISTANT PIGEONS**

by

**KATHLEEN DEKKER CURWEN
B.A., Montclair State College, 1971**

A THESIS

**Submitted to the University of New Hampshire
In Partial Fulfillment of
The Requirements for the Degree of**

**Doctor of Philosophy
Graduate School
Department of Biochemistry**

May, 1976

This thesis has been examined and approved.

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TO MY PARENTS

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Abstract

Aortic Glycosaminoglycans in Atherosclerosis-Susceptible and-Resistant Pigeons

Alterations in aortic glycosaminoglycans (GAGs) during atherogenesis may be responsible for lipid accumulations and lesion progression.

White Carneau (WC) pigeons provided a unique system for analysis of aortic GAGs since spontaneous atherosclerotic lesions in WC closely resemble those in human aortas, and, in addition, are highly predictable in terms of site specificity and sequence and rate of pathogenic progression. Since changes in GAGs occur as a result of aging, aortas from atherosclerosis-resistant Show Racer (SR) pigeons served as controls for the aortas from atherosclerosis-susceptible WC to permit discrimination between changes due to normal aging and those due to pathological involvement in WC.

Glycosaminoglycans from uninvolved thoracic aortas and from the celiac bifurcation, a site predisposed to lesion formation, of WC pigeons were isolated, separated, quantitated and compared with those from similar sites in SR. The following four ages were examined: hatching (one-day old); weaning (six-weeks old); and ages corresponding to initial ultrastructural and biochemical atherogenesis (four to six months) and moderate atherosclerosis (two to three years).

Thoracic aortas of both breeds contained similar amounts and proportions of hyaluronic acid (HA), heparitin sulfate (HS), dermatan sulfate (DS), chondroitin 4-SO₄ (Ch4-SO₄) and chondroitin 6-sulfate

(Ch6-SO₄) at corresponding ages, and showed nearly identical profiles for individual and total GAGs as a function of age. Thoracic aortas of both breeds contained more total GAG but proportionately less HS than celiac segments at each age examined.

Celiac sites of WC contained more total GAG than corresponding SR sites at all four ages. White Carneau after six months of age also showed decreased HS and Ch4-SO₄ content and Ch4SO₄:Ch6-SO₄ ratios but increased amounts of Ch6-SO₄, when compared with corresponding SR celiac foci. In addition, WC contain a variant type of HS in preatherosclerotic and moderately atherosclerotic foci which is not found in thoracic sites of either breed or in SR celiac foci at any age.

These findings are interpreted to explain atherosclerosis susceptibility and resistance in pigeons; to differentiate changes in aortic GAG content occurring with age from those due to atherogenesis; and to suggest a role for GAGs in the pathogenesis of atherosclerosis.

INTRODUCTION

Connective tissue is described by Meyer (1969) as the largest organ in the body, and one in which the cells are minor components relative to extracellular materials. The extracellular macromolecules are referred to collectively as ground substance or matrix, and supply mechanical support and protection for those cells which have elaborated them. The realization that the matrix macromolecules (proteoglycans, collagen, elastin, reticulin and glycoproteins), in addition to the cells which they surround, are biologically active, opened a new era in connective tissue research (Mathews, 1975). This research was aimed at determining the structure, functions and metabolism of component matrix macromolecules and at describing effects of these properties on the normal and pathological physiology of connective tissue. However, with the exception of glycosaminoglycans (GAGs), which constitute the largest group of proteoglycans in ground substance, the characteristics and functions of most matrix components are poorly understood (Bertelsen, 1968).

Glycosaminoglycans in different connective tissues seem to show characteristic profiles which change qualitatively and quantitatively during differentiation, aging and disease (Wight and Ross, 1975a and Robinson, Likar and Likar, 1975). Glycosaminoglycan composition may be altered by chemical, physical or biological agents which act directly on these macromolecules or indirectly through changes in the metabolism of connective tissue cells (Velican, 1974). How these alterations in extracellular GAGS in turn affect the metabolic capacities for growth, repair and remodeling of connective tissue cells remains unclear.

Although the structure and metabolism of GAG's have been described, their content, physiochemical properties, and functions in connective tissue during aging and differentiation are not as well understood. This is particularly true in the case of vascular tissue because it is distinctly stratified, accumulates considerable amounts of foreign substances (Bertelsen, 1968), and undergoes atherosclerotic degeneration during aging (Meyer, 1969). Since physical and chemical changes taking place in aortic matrix GAGs are believed to promote atherogenesis (Mathews, 1975), a better understanding of their properties, functions and profiles becomes essential.

The structure, metabolism and many of the tentative functions assigned to connective tissue GAGs in general also describe aortic GAGs. Aortic GAGs, however, possess some properties and functions which are peculiar to vascular tissue, and which are a reflection of the types and amounts of matrix GAGs present in the aorta at any given time.

The Structure and Metabolism of Connective Tissue Glycosaminoglycans

Eight connective tissue GAGs have been identified as follows: hyaluronic acid (HA), chondroitin 4-sulfate, (Ch4-SO₄), chondroitin 6-sulfate (Ch6-SO₄), dermatan sulfate (DS), heparitin sulfate (HS), heparin, chondroitin, and kerato-sulfate (KS). Only five of these (HA, Ch4-SO₄, Ch6-SO₄, DS and HS) are found in aortic tissue. As the structures of these compounds were established, their nomenclature was revised (Jeanloz, 1960); both the old and new names for the GAG's are presented in Table I.

Each GAG consists of long, unbranched chains composed of dissaccharide

Table 1. Nomenclature of the Glycosaminoglycans.

<u>Old Names:</u>	<u>New Names:</u>
Mucopolysaccharide	Glycosaminoglycan
Chondromucoprotein	Proteoglycan
Hyaluronic Acid	Hyaluronic Acid
Chondroitin Sulfate A	Chondroitin 4-Sulfate
Chondroitin Sulfate B	Dermatan Sulfate
Chondroitin Sulfate C	Chondroitin 6-Sulfate
Heparan Sulfate	Heparitin Sulfate
Chondroitin	Chondroitin
Keratosulfate	Keratan Sulfate

repeating units in which one monosaccharide is invariably a hexosamine and the other is a hexuronic acid. All except HA and Chondroitin are sulfated, and all are highly charged polyanions due to the presence of sulfate or carboxyl groups or both (Muir, 1969). The structures of the disaccharide repeating units of the five aortic GAGs are illustrated in figure I (Muir, 1969), and their constituents in Table 2.

Extensive in vivo and in vitro studies showed that GAGs of connective tissue are synthesized locally by connective tissue cells and are extruded from them (Muir, 1964 and Wight and Ross, 1975a). With the probable exception of HA, none of the GAGs occur as free polysaccharide chains, but are attached to protein cores by specific covalent linkages (Meyer, 1969). Velican (1971) suggested that the elaboration or availability of these protein cores controls the rate of GAG biosynthesis.

The biosynthesis of GAGs involves the participation of six different glycosyl transferases (Roden, 1970). These transferases catalyze the sequential transfer of individual monosaccharide components to the growing carbohydrate chain. Heath (1971) suggested that the organization and specificity of these individual glycosyl transferases are responsible for the different carbohydrate constituents which occur in individual GAGs. Uridine diphosphate (UDP) nucleotide sugars provide the monosaccharide intermediates for the transferase reactions that build up the carbohydrate chains and also for the enzymes converting common hexose precursors to the hexuronic acids and N-acetyl hexosamines required for synthesis (Muir, 1969). The initial step in GAG assembly is the transfer of UDP-xylose to the hydroxyl group of a serine residue in the core protein. Next, two UDP-galactose units are linked to the xylosyl-protein

Hyaluronic Acid

Heparitin Sulfate

Chondroitin 4-Sulfate

Chondroitin 6-Sulfate

Dermatan Sulfate

Figure 1.
Repeating Disaccharide Units of the Aortic Glycosaminoglycans.

Table 2. Constituent Disaccharide Repeating Units of the Aortic Glycosaminoglycans.

Hyaluronic Acid:

D-Glucuronic acid β (1 $\rightarrow$ 3) N-acetyl-D-Glucosamine β (1 $\rightarrow$ 4).

(1 $\rightarrow$ 4)-O- β -D-Glucopyranosyluronic acid-(1 $\rightarrow$ 3)-2-acetamido-2-deoxy- β -D-Glucopyranose.

Heparitin Sulfate:

D-Glucuronic acid α (1 $\rightarrow$ 4) N-acetyl-D-Glucosamine-6-sulfate α (1 $\rightarrow$ 4).

(1 $\rightarrow$ 4)-O- α -D-Glucopyranosyluronic acid-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy-6-O-sulfo- α -D-Glucopyranose.

Chondroitin 4-Sulfate:

D-Glucuronic acid β (1 $\rightarrow$ 3) N-acetyl-D-Galactosamine-4-sulfate β (1 $\rightarrow$ 4).

(1 $\rightarrow$ 4)-O- β -D-Glucopyranosyluronic acid-(1 $\rightarrow$ 3)-2-acetamido-2-deoxy-4-O-sulfo- β -D-Galactopyranose.

Chondroitin 6-Sulfate:

D-Glucuronic acid β (1 $\rightarrow$ 3) N-acetyl-D-Galactosamine 6-sulfate β (1 $\rightarrow$ 4).

(1 $\rightarrow$ 4)-O- β -D-Glucopyranosyluronic acid-(1 $\rightarrow$ 3)-2-acetamido-2-deoxy-6-O-sulfo- β -D-Galactopyranose.

Dermatan Sulfate:

L-Iduronic acid α (1 $\rightarrow$ 3) N-acetyl-D-Galactosamine 4-sulfate β (1 $\rightarrow$ 4).

(1 $\rightarrow$ 4)-O- α -Idopyranosyluronic acid-(1 $\rightarrow$ 3)-2-acetamido-2-deoxy-4-O-sulfo- β -D-Galactopyranose.

core to complete the linkage region. Alternating units of UDP-hexuronic acid and UDP-hexosamine are then added to the nonreducing end of the nascent polysaccharide chain (Fransson, 1970). Sulfate is added directly to the assembled polysaccharide and not to the precursor monosaccharide units. Some sulfation is believed to occur prior to completion of mature proteoglycan chains, however (Kimata, Okayama and Suzuki, 1971). Muir (1969) suggested that more than one enzyme may catalyze the transfer of sulfate from the active intermediate, 3-phosphoadenosyl-5-phosphosulfate (PAPS) to the different GAGs.

Each phase of GAG biosynthesis occurs in a specific subcellular location: protein core formation and carbohydrate chain initiation in the rough endoplasmic reticulum; polysaccharide chain elongation by monosaccharide addition in the smooth endoplasmic reticulum; and, sulfation and packaging for excretion in the golgi apparatus (Horowitz and Dorfman, 1968 and Godman and Porter, 1960).

Once GAGs are elaborated and excreted by the connective tissue cell they do not remain static, but rather undergo turnover which is probably controlled by hormonal, metabolic, and other agents capable of stimulating degradation (Hermelin et al., 1974 and Velican, 1974). Since GAGs are normally covalently bound to protein, degradation affects both the carbohydrate and protein portions of these macromolecules. Breakdown of the GAG chain requires lysosomal enzymes of great specificity. Presumably, extracellular GAG destined for digestion is ingested by pinocytosis, gains entry into the lysosomes and is eventually degraded (Neufeld and Cantz, 1973). Following removal of sulfate, hydrolases depolymerize polysaccharide chains into oligosaccharide units. Glycosaminidases and

glucuronidases further degrade these units into constitutive monosaccharide components (Neufeld and Frattoni, 1970 and Velican, 1974). Enzymes which degrade GAGs are also present in the cytosol and may be present in the pericellular space in association with the plasma membrane or in the blood plasma which perfuses the extracellular space (Velican, 1971; Velican, 1974 and Patel and Tappel, 1971).

Molecular weights ranging between 7.0×10^3 and 1.2×10^7 are reported for GAG proteoglycans (Velican, 1971 and Velican, 1974). This wide range of molecular weight is the result of extensive variations in carbohydrate content, extent and site of sulfation, carbohydrate chain length and degree and distribution of glycan chain attachment to the protein core (Linker and Hovingh, 1975; Bhavanandan and Davidson, 1975 and Velican, 1971).

The characteristics of a particular GAG also vary considerably with species and tissue of origin (Bhavanandan and Davidson, 1975 and Velican, 1971). This type of heterogeneity is particularly common for HS, which is not a single polymeric species but rather, a family of closely related compounds (Linker and Hovingh, 1975). Heparitin sulfate composition is more complex than that of most other GAGs since it can contain N-acetyl glucosamine, glucosamine N-sulfate, glucosamine N, O-disulfate, D-Glucuronic acid, L-iduronic acid and N-acetyl glucosamine O-sulfate in different disaccharide repeating units depending upon the biological source from which it is obtained (Hovingh and Linker, 1974; Linker and Hovingh, 1973 and Taylor et al., 1973). Human umbilical cord and human aorta HS for example have low total sulfate and little N-sulfate but contain many glucuronic acid-N-acetyl glucosamine disaccharides.

In contrast, beef lung HS contains L-iduronic acid in addition to D-glucuronic acid, glucosamine N-sulfate, as well as glucosamine O-sulfate, and contains more total sulfate than human umbilical or aorta HS (Cifonelli and King, 1970 and Taylor et al., 1973). In addition, some GAGs exist as hybrid macromolecules, which are actually copolymers of more than one GAG type. For example, Malmstrom et al. (1975) have identified DS - Ch6-SO₄ - Ch4-SO₄ hybrids. Unlike proteins, GAGs are not transcribed from a code, and, thus, exhibit a great degree of structural variation (Meyer, 1969). Structural variations in turn permit a wide range of functional flexibility for the GAGs.

Like proteins GAGs possess distinctive secondary and quaternary structures. Crystalline HA exists as a double helix consisting of two identical, antiparallel, left-handed strands (Armott, Guss and Hukins, 1973 and Kirkwood, 1974). Some crosslinking was suggested to stabilize these helices (Mathews, 1975). Crystalline Ch6-SO₄ exists in three-fold or eight-fold helical molecules packed parallel to one another, possibly stabilized by cationic bridges between proteoglycan strands (Armott et al., 1973 and Kirkwood, 1974). While it is tempting to suggest that these secondary structures amplify differences in the primary structures of HA and Ch6-SO₄, Mathews (1975) pointed out that it is erroneous to assume that the conformations of GAGs in tissues are identical to those in solution or in the solid states which were examined by most investigators. Quaternary structure is displayed by some connective tissue GAGs, such as keratan sulfate and chondroitin sulfate proteoglycan monomers which are found attached to a filamentous backbone of HA at regular intervals. The proteoglycans are apparently noncovalently bound by "link proteins"

to the HA backbone (Heinegard and Hascall, 1974 and Rosenberg, Hellman and Kleinschmidt, 1975).

Properties and Functions of Connective Tissue Glycosaminoglycans

The flexible chain structure, large molecular domains and large numbers of closely adjoining, fixed anionic groups which GAGs possess endow them with distinctive physiochemical properties. Tentative functions are assigned to connective tissue GAGs based on these properties and include roles in cellular phenomenon, differentiation and organogenesis.

Cellular Phenomena

Due to their anionic nature GAGs may control the cationic environment of cells and facilitate cell association by forming Ca^{++} bridges between adjacent negatively charged surface sites (Bhavanandan and Davidson, 1975). Glycosaminoglycans may possess antigenic or autoantigenic properties and could be implicated in the pathogenesis of connective tissue diseases such as rheumatoid arthritis and atheroscleroses (Robinson et al., 1975). Contact inhibition and cell division depend upon the physical state and degree of polymerization of matrix GAGs. Robinson et al. (1975) suggested that the resting cell is firmly embedded in a viscous gel of polymerized GAGs, while the dividing cell is surrounded by a liquid zone of partially depolymerized GAGs.

Differentiation and Organogenesis

Interactions between neighboring tissues in the developing embryo that lead to differentiation and organogenesis may depend upon GAGs

associated with cell surfaces and tissue interfaces. The "extracellular matrix hypothesis" (Hay and Meier, 1974) predicts that GAGs and collagen interact with the cell surface to promote or inhibit specific metabolic pathways which lead to morphogenesis and cell differentiation. Salivary morphogenesis, for example, is dependent upon epithelial cell surface GAGs which promote cleft formation by initiating collagen fibrogenesis (Bernfield, Banerjee and Cohn, 1972). Corneal morphogenesis and neural fold invagination are triggered, in part, by GAGs present in the matrix. Acting with other matrix components, GAGs may also enable embryonic tissues to control the shape and differentiation of adjacent tissues (Hay, 1973).

The Individual Glycosaminoglycans

Along with the general functions attributed to GAGs, some tentative functions are assigned to individual GAGs.

Hyaluronic Acid. Hyaluronic Acid is especially abundant in early stages of developing, regenerating, remodeling and healing tissues (Toole, 1973 and Muir, 1964), but the HA content of all connective tissue seems to decrease during the course of maturation and aging (Meyer, 1969). Hyaluronic acid is associated with high water content and presumably maintains turgor in tissue, acting as a "shock absorber" due to its high viscoelasticity (Muir, 1964). Encapsulation of cells in a matrix rich in HA prevents the cellular interactions necessary for the onset of differentiation, but permits proliferation and migration (Toole, 1973).

Heparitin Sulfate. Heparitin sulfate is made by all connective

tissue cell types, and, unlike other GAG species, HS largely remains associated with the surface of the plasma membrane (Conrad and Hart, 1975 and Kraemer, 1971). Cell surface HS may function as a superficial store of Ca^{++} ions for mammalian cells or serve as a nonspecific "screen" between cell surface sites and immediate cell environment (Buonassisi and Root, 1975 and Kraemer and Smith, 1974). Unlike heparin, to which it is closely related in structure, HS is not a strong anticoagulant (Muir, 1964 and Muir, 1969). Heparitin sulfate does, however, activate lipoprotein lipase. The removal or substitution of N-acetyl groups in HS by N-SO₄ groups increases both its anticoagulant and lipoprotein lipase activating effects (Grossman and Cifonelli, 1962). Kraemer and Tobey (1972) reported that a specific loss of surface HS occurs just prior to mitosis. This finding raises the possibility that fluctuating quantities of HS play a role in cell division, perhaps by regulating the cell cycle. Recent reports by Satoh et al. (1973 and 1974) show that viral transformation of cells in culture produces changes in the amounts and properties of HS present.

Dermatan Sulfate. It is suggested that the prime function of DS is to aid in the extracellular formation and organization of collagen fibrils (Obrink, 1973). DS possesses a striking capacity to precipitate soluble tropocollagen macromolecules into fibrillar structures at physiological pH and ionic strength, and DS is found in association with coarse collagen fibrils in vivo (Velican, 1974). Dermatan sulfate also exerts a high affinity for Ca^{++} ions and exhibits anticoagulant, antithrombogenic and lipid clearing capacities (Velican, 1971 and Dorfman, 1963).

Chondroitin 4-Sulfate and Chondroitin 6-Sulfate. Chondroitin

4-sulfate has more anticoagulant activity than Ch 6-SO₄ (Dorfman, 1963 and Morrison and Enrich, 1973) but both the chondroitin sulfates (CSs) act as inhibitors of β -glucuronidase and hyaluronidase by sequestering the polycationic activators of these enzymes (Velican, 1971). Muir (1969) reported that the CSs are found extracellularly in association with fine collagen fibrils and that the geometry and inter-group distances of the anionic sites on the CS chains are the determinants of CS-collagen arrangements.

Properties and Functions of Aortic Glycosaminoglycans

The interest of investigators in the properties, functions and distribution of GAGs in the arterial wall is based on the knowledge that the GAGs actively participate in metabolic processes taking place in the vessel wall (Nanov, Skocheva and Georgieva, 1974). Lipid deposition is hypothesized to be an indicator, not an initiator, of pathological changes in blood vessel walls resulting from quantitative and qualitative changes in vascular GAGs (Gero et al., 1962 and Robinson et al., 1975). The arterial wall is not just an inert tube with blood components moving in and out, but rather, a complex organ reacting as a whole to injuries and displaying its own structural, functional and metabolic characteristics (Velican, 1971). Arteries are able to synthesize and degrade various types of macromolecules, utilize oxygen and nutrients, regulate lipid transport and exert mechanical and visco-elastic properties. Based upon their morphological localization and biological activities, GAGs are assigned specific roles in the aortic functions of matrix integrity, permeability, blood coagulation, ion exchange, lipid clearing, enzyme

activity and complex formation with lipoproteins and fibrinogen (Robinson et al., 1975 and Gero, Bihari-Varga and Virag, 1973).

Matrix Integrity. Wight and Ross (1975b) showed that GAGs hold adjacent collagen fibrils in register by forming "bridges" at the major period bands. GAGs also hold elastic fibers together and interconnect elastic fibers with collagen fibrils. Mathews (1975) suggested that GAGs initiate and control growth in diameter of individual collagen fibrils and collagen aggregates by reversible electrostatic and covalent bonding to the surface and interior space of collagen fibrils. Thus, aortic GAGs appear to cement together the different constituents which comprise vascular ground substance.

Morrison and Schjeide (1974) suggested that circulation, permeability, blood coagulation, disposal of accumulated plaque materials and other vital physiological processes in the arterial wall are partially controlled by GAGs. The CSs do influence the activity of certain enzymes such as elastomucase, and may influence the rate at which certain enzymes are synthesized by acting as signaling agents for messenger RNA synthesis. This idea is actually very similar to the "extracellular matrix hypothesis" described by Hay and Meier (1974). Chondroitin 4-SO₄ was proposed to influence the metabolic activities of arterial cells, provoking these cells to "reconstitute the unsound intercellular matrix" present in the atherosclerotic vessel (Morrison and Schjeide, 1974). These authors suggested that optimal levels of Ch 4-SO₄ promote lipid clearing while low levels promote lipid accumulation within arterial cells.

Permeability. In the blood vessel wall, GAGs form a continuous, 3-dimensional network of entangled glycan chains which exert permeability

phenomena called molecular sieving and excluded volume effect (Mathews, 1975 and Iverius, 1973). These features become quite significant since Velican (1973) described the nutrition of the aorta (illustrated in Figure 2) as a "one way street". Under normal conditions, plasma components pass through the endothelial layer of the aorta, through the intimal and medial matrix and are propelled into the draining adventitial lymphatics. Some plasma components are consumed by intimal and medial smooth muscle cells, while others are deposited or accumulate within the matrix. This "one way street" concept of aortic nutrition has important implications in the pathogenesis of atherosclerosis, since matrix GAGs exert a molecular sieving effect upon plasma components which filter through the blood vessel wall (Velican, 1974; Iverius, 1973 and Klynstra, 1974). Retention and concentration of plasma lipoproteins within the matrix, for example, depend upon the physical characteristics of the particular lipoprotein in question, as well as on the type and concentration of matrix GAGs. Chondroitin 6-SO₄ was called a "GAG par excellence" for regulating diffusion in blood vessels since permeability decreases as the aortic content of Ch6-SO₄ increases (Bottcher and Klynstra, 1965 and Klynstra, 1974).

When GAGs or GAG aggregates are of sufficient molecular weight, ($> 10^6$) they exert an excluded volume effect within blood vessels (Velican, 1974). Hyaluronic acid is the largest of the GAGs and is most effective at both excluding large plasma macromolecules and restricting their transport through the blood vessel wall.

Iverius (1973) extended his theories of steric exclusion and molecular sieving to explain the accumulation of lipids derived from plasma

Figure 2. Cross Section of an aorta. The lumen through which the blood flows is lined by a single layer of endothelial cells. The intimal layer is relatively thin and elastic in nature. The medial layer is composed of smooth muscle cells, fibroblasts and matrix components in alternating layers and is the thickest layer. The adventitial layer is an outer fibrous coat of connective tissue cells and matrix. Small blood vessels, the vasa vasorum, are present in this layer. They penetrate only into the outermost portion of the media.

lipoproteins during the genesis of the atherosclerotic plaque. When plasma is filtered into the arterial wall all lipoproteins, with the exception of the large chylomicra, enter the intercellular matrix of the intima. The molecular sieving effect exerted by the GAGs results in the concentration of the plasma high density (HDL), low density (LDL) and, in particular, the large very low density (VLDL) lipoproteins within the intima.

While VLDL are retained within the blood vessel wall primarily by molecular sieving, additional factors may contribute to their deposition and accumulation. Some plasma glycans increase the viscosity or swelling of LDL hydrocarbon regions. This swelling contributes to decreased catabolism and an increase in molecular sieving and steric exclusion effects on the LDL within the arterial wall (Nakashima et al., 1975). With the exception of Ch4-SO₄, which prevents conformational modification of LDL, all plasma GAG species are capable of swelling circulating plasma LDL. Whether there is a direct relationship between arterial wall Ch4-SO₄ content and plasma "protective" Ch4-SO₄ content was not examined.

Blood Coagulation. Gore and Larkey (1960) have shown anticoagulant activity in DS fractions of GAG extracts of human aortas. If DS prevents coagulation, then focal depletion of DS could explain the formation of thromboses in late arterial lesions. Chondroitin 4-SO₄ extracts of animal and vascular tissue possess more antithrombogenic activity than Ch6-SO₄ extracts (Morrison and Enrick, 1973 and Morrison and Schjeide, 1974). In contrast to the antithrombogenic effect of heparin, which is immediate and short-lived, the antithrombogenic effect of Ch4-SO₄ has a lag time of several hours and is much longer lasting. Since adsorption

of polyanionic Ch4-SO₄ to blood vessel walls, blood cells, and platelets is believed responsible for preventing thrombus formation, low arterial wall Ch4-SO₄ could produce atherogenic effects in animals and in man (Morrison and Schjeide, 1974), and these effects may be attributed to the antithrombogenic properties of Ch4-SO₄ as well as to its protective, "antischwelling" effects on LDL conformations as proposed by Nakashima et al. (1975).

Ion Exchange. Iversius (1973) showed that the negatively charged carboxyl and sulfate groups in GAGs act as ion exchangers. The GAGs are capable of reversibly binding counterions such as Ca⁺⁺ (Muir, 1964) and may restrict the passage of anions, such as lactate, through the blood vessel wall. Robinson et al. (1975) suggested that cell surface HS components selectively regulate uptake of cationic macromolecules by aortic smooth muscle cells. Pollack (1974) proposed that aortic polyanions may promote transmembrane transport of lipids by changing the micellar arrangement of the lipids, by altering plasma membrane permeability or both.

Lipid Clearing. Because of their influence on the lipolytic activity of blood vessel walls, some aortic GAGs are implicated in deposition of lipid which occurs during atherogenesis. Conflicting reports concerning GAG effects on lipoprotein lipase activity show activation by DS (Gore and Larky, 1960), HS (Grossman, Cifonelli and Ozoa, 1971) and sulfated GAGs in general (Wagh et al., 1973) or inhibition by DS and the CSs (Robinson et al., 1975). If certain GAGs aid in clearing and transporting lipid in the arterial wall, then lipid accumulation could result in arteries with insufficient quantities of these GAGs.

Enzyme Activity. Glycosaminoglycans may activate or inhibit various

enzymes by interactions between their acidic groups and the basic groups of the protein molecule. These effects depend on the isoelectric point and basic amino acid content of the particular enzyme, as well as on the local pH (Robinson et al., 1975).

Complex Formation With Lipoproteins and Fibrinogen. Lipid accumulation in the arterial wall during atherosclerosis is partly the result of filtration of plasma lipids through the wall (Bertelsen, 1968). Walton and Williamson (1968), Waters (1972), Hollander, Kramsch and Inoue (1968), and others provided experimental evidence which indicates that plasma LDL and VLDL are the most important sources of lipid found in atherosclerotic lesions.

The close morphological relationship between GAG and lipid deposits in aortas led to the proposal that matrix GAGs trap plasma LP, causing them to accumulate within blood vessel walls. Adams and Bayliss (1973) showed GAGs underlying the lipid deposits following lipid extraction of aortic plaques and Srinivasan et al. (1972) succeeded in isolating a lipoprotein-GAG complex from fatty streaks of human aortas. Although the implications of LP-GAG complexes in setting the stage for atherogenesis are obvious, the exact nature of the interactions between GAGs and LP is disputed by different investigators (Iverius, 1972; Berenson et al., 1971 and Levy and Day, 1970).

In addition to the selective retention of plasma LP in the matrix proposed by Iverius (1973), several investigators suggested that aortic GAGs render LPs insoluble by chemically combining with them in the arterial wall. Berenson et al. (1971) and Amenta and Waters (1960) suggested a type of electrostatic binding which involves the formation of Ca^{++} bridges

between negatively charged sulfate groups of the GAG and negatively charged phosphate groups of the phospholipids in VLDL and LDL. Srinivasan et al., (1970) showed the order of affinity of GAGs for LDL or VLDL in the presence of Ca^{++} at pH 7.4 to be as follows: $\text{HS} > \text{Ch6-SO}_4 > \text{Ch4-SO}_4 > \text{DS}$.

Divalent cation participation in GAG-LP complex formation was strongly contradicted by Iverius (1972), Nishida and Cogan (1970), and Day and Levy, (1975). Partial delipidation of LP with phospholipase C or the addition of EDTA to reaction mixtures of LP and GAGs does not prevent formation of complexes. In addition, the involvement of phosphate groups in the interactions seemed unlikely, since GAGs do not bind HDL despite the high phospholipid content in this class of LP. The ability of GAGs to complex with LP depends upon the degree and site (N- or O-SO₃) of sulfation and the primary, secondary and quaternary structure of the GAG. The N-SO₄ groups of HS (Compared to the O-SO₃ groups of the other GAGs) enhance the affinity of HS for LP (Srinivasan et al., 1970).

Iverius (1972) proposed that the apolipoprotein portion, particularly apolipoprotein B, and not the lipid portion of LDL and VLDL, interacts with GAGs. Nishida and Cogan (1970) and Levy and Day (1970) showed that as acetylation or succinylation of VLDL and LDL apolipoproteins proceeded, resulting in an increased negative charge on the LP surface, the ability of the LP to interact with GAGs decreased. Day and Levy (1975) also showed that selective modification of LDL-arginine residues with the trimer of biacetyl eliminates LDL - GAG interactions. The authors assumed that polyanionic GAGs react with only those moieties of the LDL which are positively charged, such as lysine, arginine, and leucithin and cephalin. At physiological pH (7.4) and ionic strength

(.16), DS was the only GAG which reacted with VLDL and LDL in the absence of Ca^{++} (Iverius, 1972). This selectivity was attributed to the presence of iduronic acid in DS since charge densities of DS and the CSs are almost identical. Berenson et al. (1971) suggested that in addition to electrostatic bonds between GAGs and arginine and lysine residues in the apolipoprotein, non-electrostatic binding, such as hydrogen bonding, hydrophobic interactions or dispersion forces, may contribute to complex formations as well. Bernfeld et al. (1960) identified insoluble and reversible and irreversible soluble complexes between GAGs and VLDL or LDL. Such variable results between different laboratories may be caused by differences in pH, ionic strength, or concentrations of cations and GAGs used in the studies (Bernfeld, Donahue and Berkowitz, 1957). Furthermore, results of in vitro studies may not be representative of in vivo GAG-LP interactions.

A second serum component implicated in atherosclerotic lesion formation is fibrinogen (Walton and Williamson, 1968). Advanced lesions are often enlarged by thromboses of fibrinogen and platelets as well as by further lipoprotein accumulation (Watts, 1971). Glycosaminoglycans can sequester fibrinogen into a less metabolically active state by forming fibrinogen-GAG complexes (Berenson et al., 1971). Fibrinogen is capable of interacting with GAGs in vitro not only on the acidic but on the alkaline side of its isoelectric pH, and fibrinogen-GAG complexes are usually more soluble than corresponding LP-GAG complexes (Robinson et al., 1975).

Aortic Glycosaminoglycan Content

Many attempts have been made to correlate qualitative and quan-

titative changes in aortic GAGs with the processes of aging and atherogenesis, and to explain these processes in terms of GAG-mediated changes in arterial wall matrix integrity, permeability, diffusion, mechanical properties, enzyme activities and lipoprotein and fibrinogen accumulation (Kumar et al., 1967a; Murata, 1974; Stefanovich and Gore, 1967; Nanov et al., 1974 and Farquhar and Dunstone, 1968). Unfortunately, decades of efforts by these and other workers resulted in a mass of contradictory data which yields no consistent patterns or no satisfactory explanations for the role of GAGs in aging or in atherogenesis.

General Conclusions

Taking literature discrepancies and experimental variables into account, one can still make some general observations concerning aortic GAG content during maturation and atherogenesis. Regardless of the species of origin or the type or portion of aorta studied, most aortic tissue contains approximately 0.5 - 2.0% GAG on a dry defatted weight basis or 0.1 - 0.5% on a wet-weight basis (Stefanovich and Gore, 1967).

Thoracic aortas invariably contain more total GAG than corresponding abdominal aortas in all species (Robinson et al., 1975; Souadjian, Kottke and Titus, 1969 and Stefanovich and Akiyama, 1970). These findings were explained by Nanov et al. (1974) on the basis of aortic muscularity and elasticity. Three types of vessels, elastic, muscular and hybrid are distinguished. Thoracic aortas are representative of elastic vessels which contain the most GAGs; hybrid segments of vessels which are transitional between elastic and muscular vessels contain less GAGs; and abdominal aortas, which are representative of muscular vessels contain

the least amounts of GAGs. This means that aortic GAG content decreases with distance from the heart.

The types, as well as quantity, of GAG's found in thoracic aortas differ from those of abdominal aortas. Heparitin sulfate comprises a much greater proportion of the GAGs in abdominal aortas than in thoracic aorta segments, while the converse is true for DS. Chondroitin sulfate contents of thoracic and abdominal aortas are of the same proportion (Farquhar and Dunstone, 1968; Stefanovich and Akiyama, 1970 and Engel, 1971).

When analyzed separately, the intimal layer of both thoracic and abdominal aortas always contains more GAGs (by weight) than the medial layer (Klynstra et al., 1967; Murata, 1974 and Robinson et al., 1975).

Correlations between amount of aortic GAG and species susceptibility to atherosclerosis are consistently found (Robinson et al., 1975; Gero et al., 1973; Mullinger and Manley, 1969 and Gardias, Picard and Hermelin, 1973). Atherosclerosis-susceptible species (e.g. pigeons, rabbits, man) contain greater amounts of aortic GAGs than atherosclerosis-resistant species (e.g. rats, mice, hamsters). Nanov et al. (1974) showed that susceptible species in general have aortas which are more elastic (less muscular) in nature than are the aortas of resistant species. These elastic vessels contain more total GAGs than muscular vessels.

In addition, Gardias et al. (1973), Hermelin et al. (1974) and Mullinger and Manley (1969) reported lower levels of HS in medial and intimal layers of the thoracic aortas of atherosclerosis-sensitive species when compared with resistant species. Chondroitin sulfate is the major GAG reported in the susceptible species, while HS is the major GAG in

resistant species.

Glycosaminoglycans in Aging

Attempts made to distinguish changes in aortic GAG occurring with age from those related to atherogenesis show increases (Nakamura et al., 1971 and Bertelsen, 1968), decreases (Velican, 1974), and no net changes (Kumar et al., 1967a), in content with age. Berenson et al. (1971) described these changes as generally being of small magnitude. Schmidt and Dmochowski (1964a) for example, reported the percent GAG as uronic acid/g dry defatted and decalcified aortic tissue to be 2.45 in newborn humans and 2.50 and 2.68 in normal adults. These changes in percent uronic acid content are smaller for aging than for those occurring due to atherosclerosis. (Values reported in the same experiment were 2.88 in slightly atherosclerotic vessels and 3.22 and 3.48 in distinctly atherosclerotic vessels.) Kumar et al. (1967a) showed an increase in aortic GAG content until the third decade of life, when full aorta size was reached. After the third decade, a gradual decrease in human aortic GAG content occurred. Variations in individual GAG profiles with aging are also controversial. Most reports (Kumar et al., 1967a; Muir, 1964 and Velican, 1974) show a decrease in HA expressed as mg uronic acid/g dry defatted tissue after the first decade in human aortic tissue, and at comparable ages in other animal species. One report on rat aortas (Vijayakumar, Leelama and Kurup, 1975), however, showed a decrease in HA aortic content from weanling until six months and then a subsequent increase. While Nakamura et al. (1971) reported no change in the relative proportions of the sulfated GAGs with age in humans, Kumar et al. (1967a)

reported increases in aortic DS, HS and CSs in humans until the second or third decade and then subsequent decreases.

Glycosaminoglycans in Atherogenesis

The tendency for GAG to increase in the arterial wall with the onset of fatty spots and streaks and then to decrease with further transformation of these areas into fibrous plaques and complicated lesions is repeatedly stressed (Velican, 1974; Kumar et al., 1967a; Kumar et al., 1967b; Wagh et al., 1973; and Hollander et al., 1968). One such analysis carried out by Bottcher and Klynstra (1962) showed mg GAG/g dry defatted tissue as 18.2 in undiseased vessels, 20.2 in fatty spots and streaks, 18.7 in the fibrous plaques and 16.5 in the complicated lesions of atherosclerosis.

The progression of the disease is also marked by altered profiles of the individual GAGs. HA is present in low quantities (less 10% total GAG) and decreases during atherogenesis (Schmidt and Dmochowski, 1964a; Nakamura et al., 1971 and Klynstra et al., 1967). No consistent profile for HS emerges during atherogenesis. Different reports show increases, decreases, no change, increases followed by decreases, etc. Dermatan sulfate content is elevated in fatty spots and streaks compared to uninvolved aortic segments, but is significantly decreased in fibrous plaques. (Velican, 1974; Vijayakumar et al., 1975 and Kumar et al., 1967b).

The presence, content, and profile of one GAG, Ch4-SO₄ in aortic tissue during atherogenesis is uncertain, because Ch4-SO₄ closely resembles Ch6-SO₄ in structure and is rarely separated from this isomer in quantitative studies on aortic GAG content. Velican (1974) and Murata (1974) report that Ch6-SO₄ is the major GAG component of the arterial wall

(greater than 50% of total GAG) and that it increases in quantity until advanced lesion stages, when it declines gradually. The proportions of the three isomers, DS, Ch4-SO₄ and Ch6-SO₄ in atherosclerotic tissue are usually 1:1:2.5 (Murata, 1974).

Explanations For Literature Discrepancies

Kumar et al. (1967a), Robinson et al. (1975) and Murata (1974) explained discrepancies found in the literature in terms of the variables which are a part of these studies. Considerable variations in content of GAGs from different species, from individuals within the same species, and even from different layers and segments of the same aorta are reported. Pooled samples are often grouped without regard for age, race, sex and anatomy. Bias in selection and mixing of sample material often results in pooling uninvolved aorta segments with lesion segments or in mixing different lesion types. Attempts to show distinctions between changes due to aging and those due to atherogenesis are rarely made. Differentiation of a large artery, such as the aorta, may not be completed before the onset of pathological changes, so that even when such attempts are made, uncertainties are introduced because of simultaneous degeneration and maturation processes (Mathews, 1975).

In addition, different methodologies used by various investigators make comparisons of results difficult since quantitative isolation and adequate separation and quantitation techniques are not always employed (Greiling, 1974). Both histological and biochemical approaches are used to study aortic GAG content. Early chemical methods for measuring GAG content were based on total hexosamine, uronic acid or sulfate determinations.

Improvements in GAG extraction, fractionation, electrophoresis, staining, and enzymatic degradation allow more critical quantitation and identification of individual GAGs, but difficulties with these techniques still exist. Ion exchange and cellulose column chromatography, for example, do not provide sharp separations of individual GAG fractions (Tanaka and Gore, 1966) and most electrophoretic techniques separate GAGs into only two or three bands (Breen et al., 1970 and Saxena and Nagchaudhuri, 1968). In addition, many techniques are not sensitive enough for estimating absolute amounts of submicrogram quantities of GAGs. Separation of the individual CS isomers was impossible until the recent commercial availability of Chondroitinases and Chondro-sulfatases. Even with the use of these specialized enzymes the separation of individual CSs is difficult in aortic tissue where different CS glycan chains attach to the same peptide core and the glycan chains themselves are often copolymeric structures. Bovine aortas, for example, contain GAGs in which DS disaccharides are located in the same chain with Ch6-SO₄ and Ch4-SO₄ disaccharide units (Ehrlich, Radhakrisnamurthy and Berenson, 1975 and Malmstrom et al., 1975). Finally, results are further complicated by the expression of analytical data on the basis of protein, dry weight, wet weight, or dry defatted weight.

The Present Study

Since early atherosclerotic lesions may be both preventable and reversible (Dalferes et al., 1971), it is important to understand their pathogenesis and to define the initiating factors. Alterations in aortic GAGs are believed to play a role in lipid accumulation which is the key

pathological feature of atherosclerosis. Therefore, the object of the present study was to analyze the GAGs present in aortas of atherosclerosis-resistant and atherosclerosis-susceptible breeds of pigeons at ages corresponding to preatherosclerotic and early atherosclerotic stages. GAGs from uninvolved thoracic aortas and from the celiac bifurcation (a site predisposed to atherosclerotic involvement) in aortas of susceptible White Carneau (WC) pigeons were compared with GAGs from similar sites in resistant Show Racer (SR) pigeons. Quantitative and qualitative comparisons were made at the following ages: one-day old (hatching), six-week old (weanling), four-to six-month old (first ultrastructural and biochemical atherogenesis), and two-to three-year old (moderate atherosclerosis).

White Carneau pigeons provide a unique system for analysis of aortic GAGs since spontaneous atherosclerotic lesions in WC closely resemble those in human aortas biochemically, histochemically and pathologically (Clarkson et al., 1959; Prichard et al., 1962 and Herndon et al., 1962). In addition, WC lesions are highly predictable in terms of site specificity and in the sequence and rate of pathogenic progression (Santerre et al., 1972). Because alterations in the arterial wall also occur as a result of aging, Show Racer pigeons serve as controls, to permit discrimination between changes due to normal aging and those due to pathological involvement in WC.

MATERIALS AND METHODS

Derivation of Experimental Tissue

Six-week, six-month and two-to three-year old White Carneau (WC) and Show Racer (SR) pigeons were obtained from Palmetto Pigeon Plant (Sumter, South Carolina). Birds were housed in fly-coops prior to shipment and were fed a mixture of yellow corn, wheat, peas, kafir and Palmetto Health Grit. One-day old pigeons were obtained from WC and SR colonies maintained at the University of New Hampshire. These colonies were originally derived from Palmetto Pigeon Plant Stock of the same lines as described by Clarkson, et al. (1959).

Birds were sacrificed by exsanguination, and upper thoracic aortas from the arch to just below the celiac bifurcation were removed, placed in ice-cold Hanks' Balanced Salt Solution with Glucose, and dissected free of blood, extraneous material and adventitia. Thoracic portions and celiac foci as described by Santerre et al. (1972) were separated and stored in air-tight glass vials at -20° C until sufficient tissue pool sizes were obtained to begin isolation of glycosaminoglycans.

Isolation of Glycosaminoglycans from Pigeon Aortas

Glycosaminoglycans were reported to make up approximately 0.1 % of aortic tissue on a wet weight basis (Stefanovich and Gore, 1967). Since 250 ug GAGs were necessary for analytical procedures, six thoracic segments from six-week, six-month, or two-to three-year old birds (each weighing 45 - 100 mg), 30 celiac foci from six-week, six-month or two-to three-year old birds (each weighing 5 - 10 mg), 40 thoracic segments from one-

day-old birds (each weighing 5-7 mg), and 300 celiac foci from one-day old birds (each weighing 0.4-1 mg) constituted optimal pool sizes of 250 mg or more. Approximately equal numbers of male and female aortas were used in each pool in order to compensate for any differences in GAG content due to sex.

The GAGs were isolated from frozen aortic tissue using a modification of the isolation procedure of Mankin and Lipiello (1971). The aortic tissue pool was weighed, minced into pieces less than 1 mm-square with scissors and soaked in 30.0 ml of absolute ethanol in a screw cap vial (25 x 95 mm) at room temperature in order to remove water and lipid. After 24 hr the ethanol was partially removed with a Pasteur pipette, and the remainder removed under vacuum prior to lyophilization of tissue. The lyophilized residue was transferred to a weighing dish, and dry defatted weight determined. One ml of 1.0 M phosphate buffer, pH 7.0, was added to the tissue in a screw cap vial and hydrolytic enzymes were destroyed by heating the vial in a boiling water bath for 20 minutes. The vial was then cooled, and a solution of 2.0% pronase (Calbiochem) in 0.2 M tris (hydroxy methyl) amino methane (Tris) buffer, pH 8.0, was added to give a final concentration of 100 μ g pronase/mg dry defatted tissue. The mixture was incubated for 48 hr at 37° C. A second proteolytic digestion was performed by adding a solution of 0.2% crude papain (Sigma) in 0.1 M phosphate buffer, pH 7.0, containing 0.01 M cysteine and 0.01 M disodium ethylenediaminetetraacetate (EDTA) to give a final concentration of 5 μ g papain /mg dry defatted tissue. This mixture was incubated at 65° C for 18 hr.

After completion of the second proteolytic digestion the aortic

tissue appeared completely solubilized. This solution, along with four 0.5 ml water washings of the vial were transferred to a polyallomer centrifuge tube (Beckman, 1" x 3 1/2") which was then chilled to 4° C. Solid trichloroacetic acid (TCA) was added to give a final concentration of 5% (w/v). The tube was incubated at 4° C for 4 hr and then centrifuged at 7,000 rpm (4,200 x g) in a type 30 head in a Beckman Model L Ultra-centrifuge for ten minutes at 4° C.

Following centrifugation the supernatant was transferred to a second centrifuge tube. The TCA precipitate was washed with 1.0 ml of 5% (w/v) TCA and this solution pooled with the supernatant. Three volumes of cold 5% (w/v) potassium acetate in absolute ethanol for each volume of combined supernatant (containing the GAGs) was added to the tube, and the mixture incubated at 4° C for 16 hr. This solution was then centrifuged at 4° C at 18,000 rpm (27,000 x g) in a type 30 head for 15 minutes. The resultant GAG precipitate was successively extracted with 1.0 ml portions of absolute ethanol, ethanol: diethyl ether, (1:1, v:v) and diethyl ether and was then thoroughly air dried.

Next, 1.0 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.005 M $MgCl_2$, 25 $\mu g/ml$ deoxyribonuclease (Worthington), and 10 $\mu g/ml$ ribonuclease (Worthington) was added to the centrifuge tube, the tube capped, and incubated at 37° C for 16 hr.

Tube contents along with water washings of the tube were transferred to 3.24 cm (dry) diameter cellulose dialysis tubing (Fischer Scientific) and dialyzed against distilled water in a four liter flask at 4° C with stirring. Dialysis water was changed two or three times daily for a minimum of four days of dialysis.

Finally the GAG solution was lyophilized, and the purified, dried GAGs were dissolved in double distilled water at approximately 3 μ l water/mg dry defatted tissue. This dilution ratio was used to give a final concentration of approximately 5 μ g GAG/ μ l.

Cellulose Acetate Electrophoresis

GAGs were separated by cellulose acetate electrophoresis by a modification of the method of Stefanovich and Gore (1967). Since GAG electrophoretic patterns differ depending upon species of origin (Stefanovich and Akiyama, 1970) electrophoresis conditions were determined which would afford optimal separation of pigeon aorta GAGs.

Staining and destaining techniques were also evaluated to permit maximal visualization of bands. The following methodological parameters were examined: pH (3.25-8.1), acetate salts (Cd, Ca, Cu, Zn, Ba), buffer concentrations (0.1 M- 4.0 M), amperages (1.0 - 4.0 mA/strip), migration times (2 - 7 hr), power supplies (constant current or pulsed), stains (alcian blue, toluidine blue or acridine orange) and destaining solutions (dilute acetic acid, methanol, Fisher Decalin [decahydronaphthalene] or Gelman Sepra Clear). The best separations and quantitations were achieved with the following method.

Cellulose polyacetate strips (Gelman Sepraphore III, 1" x 6") were soaked for 15 - 18 hr at 4° C in electrophoresis buffer (0.3 M cadmium acetate adjusted to pH 4.1 with glacial acetic acid). Strips were blotted lightly on Whatman No. 1 filter paper, and GAGs (2.0 - 15.0 μ g in 2.0 μ l) were applied as a line at the origin (2.5 cm from the cathode end) with a Gelman Serum Applicator. Two Gelman Deluxe

Electrophoresis Chambers containing a total of six sample and eight standard strips were routinely run simultaneously for 4 1/2 hr with cold (ca. 20° C) tap water circulating through the chambers. A constant current of 2.0 mA/strip was supplied by a Buchler Voltage and Current D.C. power supply.

Standard heparitin sulfate (HS) was the generous gift of Dr. J. A. Cifonelli (Univ. of Chicago, Dept. of Pediatrics). Hyaluronic acid (HA), dermatan sulfate (DS), chondroitin 4-sulfate (Ch4-SO₄) and chondroitin 6-sulfate (Ch6-SO₄) were purchased from Miles Laboratories. The HS was a National Heart Institute Reference Standard, while HA, DS and Ch 4- and 6-SO₄ were Miles Super Special Grade (specified pure by infrared spectroscopic analysis). All standards were electrophoretically homogeneous under the conditions described previously.

Following electrophoresis, four bands were visualized when strips were floated on, and then immersed for 10 minutes in, an alcian blue stain containing 1.0g Alcian Blue 8GX (Allied Chemicals), 100 ml absolute ethanol and 50 ml glacial acetic acid/liter. Background destaining was achieved by soaking strips for 60 minutes in 7.5% (v/v) acetic acid with five changes of the destaining solution. Strips were then blotted on Whatman No. 1 filter paper, flattened on glass slides, and dried under a stream of warm air from an Oster Airjet Hair Dryer.

Quantitation of bands was accomplished by in situ densitometric scanning of strips. A Canalco Model K Densitometer was used with the following specifications: yellow filter (530 nm), 0-1 linear optical density scale, 0.02 sensitivity, slow scan speed, and a chart speed of 3.0 inches/minute.

Samples were evaluated by comparisons with standard curves prepared by plotting known GAG concentrations vs. area under the corresponding densitometric curves. These areas were determined by planimetry using a Keuffel and Esser Compensating Polar Planimeter.

The resulting four bands were identified by Stefanovich and Gore (1967) and Picard, Gardias and Esquive (1970), in similar cellulose acetate electrophoresis systems, as: HA, HS, DS and Ch4-SO₄/Ch6-SO₄ in order of increasing electrophoretic mobility. In order to show that the identities of pigeon and standard GAG bands corresponded to the bands reported by these authors, three methods were employed. First, standard GAG preparations were run electrophoretically one at a time and electrophoretic mobility demonstrated as: Ch6-SO₄ = Ch4-SO₄ > DS > HS > HA. Second, individual standard GAGs were added one at a time to aliquots of pigeon GAG mixtures and the identities of the overloaded band of pigeon GAG plus carrier were determined. In this way the electrophoretic mobilities of pigeon GAGs were also shown to be Ch6-SO₄/Ch4-SO₄ > DS > HS > HA. Finally the identities of the GAG bands were determined enzymatically by an adaptation of the methods of Picard et al. (1970). In this procedure aliquots of sample and of standard GAG mixtures were electrophoresed before and after enzymatic hydrolysis by leech hyaluronidase (E.C.4.2.99.1), (Biotrics, Inc.) chondroitinase - ABC (E.C.4.2.2.4) (Miles), chondroitinase-AC (E.C.4.2.2.5) (Miles) or heparitinase (E.C.3.2.1.) (the generous gift of Dr. Alfred Linker, Veteran's Administration Hospital, Salt Lake City, Utah). Leech hyaluronidase is specific for HA (Yuki and Fishman, 1963; Linker et al., 1960), heparitinase is specific for HS (Hovingh and Linker, 1970), chondroitinase-ABC hydrolyzes HA, DS, Ch4-SO₄ and Ch6-SO₄

(Yamagata et al., 1968) and chondroitinase-AC hydrolyzes HA, Ch4-SO₄ and Ch6-SO₄.

For each enzymatic degradation 10.0 μ l of sample or standard GAG solution, (containing 2.5 - 5.0 μ g/ μ l total GAG) was incubated with one enzyme and appropriate buffer in small glass stoppered test tubes (13 x 40 mm). For leech hyaluronidase digests, 0.45 mg of enzyme was dissolved in 4.5 ml of cold (0° C), double-distilled water. Ten μ l of this enzyme solution was added to 10.0 μ l of GAG mixture in 0.5 ml of citrate-phosphate buffer, pH 5.6, (McIlvaine, 1921) once every hour for five hours (Balazs, Personal Communication), and the reaction mixture was incubated at 37° C for 16 hr. For chondroitinase digests, 0.06 units of chondroitinase-AC or 0.02 units of chondroitinase-ABC in double-distilled water was added to 10.0 μ l of GAG mixture with 15.0 μ l of enriched Tris buffer (Saito, Yamagata and Suzuki, 1968). This buffer was composed of 3.0 g Tris 2.4 g sodium acetate, 1.46 g NaCl and 50.0 mg bovine serum albumin (Sigma) in 100.0 ml double-distilled water adjusted to pH 8.0 with dilute HCl. Reaction mixtures were incubated at 37° C for 16 hr. For heparitinase digests a small amount of heparitinase (approximately 10.0 μ g) was dissolved in 3.0 ml of 0.1M sodium acetate, pH 7.0. Ten μ l of this solution was added to 10.0 μ l of GAG mixture and incubated at 37° C for 24 hr (Linker, Personal Communication). Blanks (mixtures with buffer and GAGs but no enzymes) were run simultaneously with digests.

After digestions were completed, tubes containing reaction mixtures were lyophilized and then redissolved in 5.0 μ l double distilled water. A 2.0 μ l aliquot of this solution was electrophoresed as described previously to confirm the identity of the four electrophoretic bands as HA, HS, DS,

and Ch4-SO_4 - Ch6-SO_4 in order of increasing mobility.

Enzymatic Determination of Isomeric Chondroitin 4-and 6-Sulfates

The separation and quantitation of Ch4-SO_4 and Ch6-SO_4 which was not accomplished electrophoretically, was achieved by measuring the borate-catalyzed Elson-Morgan reaction color of GAG samples (Strominger, Park and Thompson, 1959; Schmidt and Dmochowski, 1964b). The analysis is based on the observation made by Matthews and Inouye (1961) that oligosaccharides containing free or $\text{O-SO}_3\text{H}$ substituted N-acetylhexosamines in the six position yield full color, while those substituted in the four position cause an almost complete suppression of color formation. The production of color in this test is dependent upon two processes: the formation of an intermediate compound probably a disubstituted oxazole (or oxazoline), by heating the acetylhexosamine with alkali, and the reaction of this intermediate with p-dimethylaminobenzaldehyde in acid (Reissig, Strominger and Leloir, 1955 and Morgan, 1946). N-acetylhexosamines substituted in the four position are unable to form the intermediate compound (White, 1940; Cifonelli and Dorfman, 1958; Morgan, 1946 and Kuhn, Gauke and Baer, 1954).

N-acetylhexosamine-containing disaccharides are released from polymeric GAG mixtures by the actions of chondroitinase-AC and chondro-4-sulfatase (E.C.3.1.6.4), (Miles). These enzymes were purified and characterized by Yamagata et al. (1968). Chondroitinase-AC catalyzes the reactions:¹

Chondro-4-sulfatase catalyzes the reaction:

No activity was shown by chondroitinase-AC on DS or HS substrates, nor was activity shown by chondro-4-sulfatase on Di-6SO₄ (Yamagata et al., 1968).

Analysis of Ch4-SO₄ and Ch6-SO₄ was carried out in a two-step reaction procedure. In the first step the concentration of Ch6-SO₄ in a sample aliquot was measured by adding chondroitinase-AC to the reaction mixture. Colored products were given only by Ch6-SO₄ and HA in the subsequent Elson-Morgan reaction. Since the concentration of HA was determined previously by cellulose acetate electrophoresis, its contribution to total color was subtracted out by running appropriate standards. The standards contained the known concentration of HA and varying concentrations of Ch6-SO₄. The concentration of Ch6-SO₄ in the sample was determined by comparison to standard curves of concentration of Ch6-SO₄ vs. absorbance at 585 nm.

In the second step both chondroitinase-AC and chondro-4-sulfatase were added to the sample aliquot. Elson-Morgan colored products were formed by Ch6-SO₄, HA and Ch4-SO₄. The contributions of Ch6-SO₄ and HA to total color were subtracted out by again running appropriate standards, this time with known concentrations of Ch6-SO₄ and HA and varying concentrations of Ch4-SO₄. The concentration of Ch0-SO₄ disaccharides liberated

¹The abbreviations used are: $\Delta \text{Di-4SO}_4$, $\Delta \text{Di-6SO}_4$, and $\Delta \text{Di-OSO}_4$, the disaccharide repeating units of Ch4-SO₄, Ch6-SO₄ and HA respectively, as shown in Table 2.

were determined quantitatively by comparison to standard curves of concentration of Ch4-SO_4 absorbance.

The details of the reaction procedures were as follows. All reaction mixtures were prepared in glass stoppered test tubes (13 x 40 mm). The following solutions were contained in a total volume of 75 μl : 15 μl of enriched Tris buffer (3.0 g Tris, 2.4 g sodium acetate, 1.46 g NaCl, 50.0 mg bovine serum albumin [Sigma] in 100.0 ml double distilled water adjusted to pH 8.0 with dilute HCl), test sample or standard GAG solution containing 1.0 - 20.0 μg of the individual isomer to be analyzed, and either 0.06 units of chondroitinase-AC or 0.06 units of chondroitinase-AC with 0.01 units of chondro-4-sulfatase. The blank was prepared without the enzymes, and all reaction mixtures were incubated for 16 hr at 37°C.

After incubation, 75 μl of a 5% (w/v) tetraborate buffer, pH 9.0, (adjusted with boric acid) was added to each tube. Tubes were mixed vigorously in a Vortex Genie (Fisher) for thirty seconds, placed in a boiling water bath, heated for exactly seven minutes, and then cooled in ice water. To each cold reaction mixture was added 600 μl of glacial acetic acid and 250 μl of paradimethylaminobenzaldehyde (DMAB) reagent (1.6 grams of DMAB [Eastman Kodak] dissolved in 9.5 ml of glacial acetic acid and 0.5 ml HCl). Tubes were mixed vigorously for thirty seconds in the Vortex-Genie, and color was allowed to develop at room temperature.

Quantitation was achieved by reading the absorbance of reaction mixtures at 585 nm in a Beckman DU Spectrophotometer between 90 - 120 minutes after addition of the DMAB reagent to the tubes. A linear relationship was shown for concentration of isomer present vs. the

absorbance in the range of 1.0 - 20.0 μg of individual isomer.

Determination of Total Uronic Acid

Uronic acid assays of sample aliquots were performed (whenever sample size permitted) in order to confirm the validity of total GAG values obtained by the combined electrophoresis and Elson-Morgan techniques. Since color yield per mole of uronic acid is not the same for all GAGs (for example DS color yield is 15% higher than HA) and since protein and hexoses are interfering chromagens (Svejcar and Robertson, 1967), the uronic acid assay is not as accurate as the combined techniques for quantitating individual GAGs present in aliquot mixtures.

Total uronic acid was determined by the Orcinol Procedure, in which uronic acid is converted to a furfural-type chromagen after heating with strong acid (Davidson, 1966). Ten μl of sample or standard GAGs (identical to those used in electrophoresis and Elson-Morgan color reactions) made up of equal amounts (w/v), of each of the five GAG standards, containing 1.0 - 15.0 $\mu\text{g}/\mu\text{l}$ were added to 990.0 μl of double-distilled water and 3.0 ml of orcinol reagent in a test tube (16 x 150 mm). Orcinol reagent was prepared by adding 40.0 mg orcinol (Fisher) to 15.5 ml HCl and 1.0 ml of 1.5% (w/v) ferric chloride. Tubes were capped with marbles, heated for 40 minutes in a boiling water bath, cooled to room temperature and the absorbance read immediately at 600 nm in a Bausch and Lomb Spectronic 20 Spectrophotometer. Sample uronic acid was quantitated by comparison with standard curves obtained by plotting standard GAG concentration vs. absorbance. A linear relationship was shown for uronic acid vs. absorbance in the range of 10.00 - 150.0 μg

GAG.

Range of Methods

The useful ranges of the methods used to analyze GAGs appear in Table 3. The maximal amounts of HA, HS, or DS which could be measured by in situ densitometric scanning of cellulose acetate strips were limited by the capacity of the polyacetate membranes to retain alcian blue-GAG complexes. An excess of the complex on the strip was indicated by irregularly shaped and colored bands which were blotched and cracked in appearance. Overloading the strips with sample also resulted in overlapping of adjacent bands. Below the lower limits indicated for HA, HS and DS (Table 3), the bands were not intense enough for densitometric detection. Since the affinity of individual GAGs for alcian blue is different, the range of each GAG shows a different lower and upper limit. Breen et al. (1970) and Saxena and Nagchuadhuri (1968) have also indicated that the amount of alcian blue bound per disaccharide repeating unit is different for each GAG, and is a function of the charge density of the particular disaccharide unit.

Coefficients of determination for HA, HS and DS represent means of the squares of the correlation coefficients of twenty-five standard curves for each GAG. These coefficients are a measure of how well a regression line fits the sample data, with a value of 1.00 being a perfect fit (Freese, 1967).

The minimal amounts of $\text{Ch}_4\text{-SO}_4$ measurable by the Elson-Morgan colorimetric determination of hexosamine were determined by the sensitivity of the spectrophotometer. The maximal amounts were limited by concentrations of reagents. Above 20.0 μg of individual isomer there was not

Table 3. Ranges for Quantitation of Glycosaminoglycans.

GAG	Lower Limit μg	Upper Limit μg	Method	Coefficient of Determination
HA	.25	3.0	cellulose acetate	.987
HS	.20	2.5	cellulose acetate	.990
DS	.15	2.0	cellulose acetate	.988
Ch 4-SO ₄	1.0	20.0	Elson - Morgan	.993
Ch 6-SO ₄	1.0	20.0	Elson - Morgan	.997
total GAG	10.0	150.0	Orcinol	.997

sufficient enzyme present in the reaction mixtures to insure complete degradation of CS polysaccharides into disaccharide units during the 16 hr digestion period. Saito et al. (1968) reported departures from linearity in optical density readings taken at disaccharide concentrations greater than 20.0 μg isomer per reaction mixture. The mean coefficients of determination for twenty-five standard curves for Ch4-SO₄ and for Ch6-SO₄ show the linear relationships between concentration of each CS and optical density at 585 nm within the specified ranges (Table 3).

The lower and upper limits of total GAGs which could be measured by the orcinol colorimetric determination for uronic acid were limited by spectrophotometer sensitivity. The coefficient of determination in this case represents the means of the squares of the correlation coefficients of four standard curves (Freese, 1967).

Accuracy and Precision of Methods

The accuracies of the cellulose acetate electrophoretic procedure and the Elson-Morgan colorimetric method were determined by repeated analysis of varying amounts of standard GAGs. Least squares regression analyses were applied to sets of data points from twenty-five standard curves for each GAG over the ranges of concentrations specified in Table 3. (See appendix Tables 1-5). Predicted values for one standard value in each standard curve considered as unknown determined from least squares regression lines were compared to actual values to obtain residual errors for each GAG. Accuracies of the methods were then established by determining confidence limits (Freese, 1967) for the residual errors of each standard GAG. Accuracy of the cellulose acetate method was $\pm .03 \mu\text{g}$

for HA, $\pm .05 \mu\text{g}$ for HS and $\pm .05 \mu\text{g}$ for DS at the 99% confidence level, and the accuracy of the Elson-Morgan method was $\pm .07 \mu\text{g}$ for both Ch4-SO₄ and Ch6-SO₄ at the 99% confidence level.

Replicate analysis of five paired aliquots of HA, HS and DS, and three paired aliquots of Ch4-SO₄ and Ch6-SO₄ were performed routinely. Confidence limits were established for the differences of replicate analyses from the mean values for a series of twenty-five replicate analyses as an estimate of the precision of the method used (See Appendix Tables 6-10). The precision of the cellulose acetate method was $\pm .06 \mu\text{g}$ for HA, $\pm .09 \mu\text{g}$ for HS and $\pm .06 \mu\text{g}$ for DS at the 99% confidence level. The precision of the Elson-Morgan method was $\pm .03 \mu\text{g}$ for Ch4-SO₄ and $\pm .04 \mu\text{g}$ for Ch6-SO₄ at the 99% confidence level.

Experimental Design

The basic experiment was designed as a 4 x 2 x 2 factorial in a randomized block design. A diagram of a single replicate appears below.

Breed	White Carneau		Show Racer	
Age	Thoracic Aorta	Celiac Focus	Thoracic Aorta	Celiac Focus
1 day	x	x	x	x
6 week	x	x	x	x
6 month	x	x	x	x
2-3 years	x	x	x	x

Each x represents the total and the 5 individual ug GAG/mg of dry defatted tissue for one experimental unit. Most replicate experiments were performed in a given time period during which all treatment combinations were analyzed prior to beginning the next replicate. Two to seven separate replicates of thoracic portions and one or two replicates of celiac portions of each breed at each age were analyzed.

RESULTS

Qualitative Analysis

Pigeon and Standard Glycosaminoglycan Electrophoretic Profiles

Cellulose acetate strips of GAG standards and WC and SR thoracic GAG samples at all four age groups run under the same conditions are illustrated in Figure 3. Both WC and SR thoracic GAGs show the same electrophoretic profiles at corresponding ages, but one-day old profiles show wider, more diffuse DS and CS bands than profiles from the three older age groups. However, pigeon aorta profiles are not identical to those of the standards. Pigeon aorta HA and DS migrate the same distance from the origin as standard HA and DS respectively, but the pigeon aorta HS and CS migrate more slowly than standard HS and CS bands respectively. In addition, the pigeon DS and CS bands (particularly from one-day samples) are wider and more poorly resolved than the standard DS and CS bands. Figure 4 illustrates these features more clearly via densitometric tracings of electrophoretically separated standards and thoracic aorta GAGs from one-day and six-month WC. Since the charge density of aortic GAGs varies with the species studied (Gardias et al., 1973), so do the electrophoretic profiles. The biological sources of the standards used in the present study were as follows: HA - human umbilical cord; HS - beef lung; DS - pig skin; Ch4-SO₄ - whale cartilage; and Ch6-SO₄ - shark cartilage. Electrophoretic analysis of aortic GAG from fifteen species in a system similar to the one used in this study showed several different electrophoretic profiles. In addition, in pheasants and chickens DS bands could not be separated from CS bands

Figure 3. Cellulose acetate electrophoresis strips showing standard and WC and SR thoracic aorta GAG separations. Emb (embryonic) indicates one-day old birds, O is the origin and 4/6 the Ch 4-SO₄/Ch 6-SO₄ band.

Figure 4. Densitometric tracings of electrophoretically separated GAGs. a) one-day old thoracic WC
b) six-month old thoracic WC
c) standards

(Stefanovich and Akiyama, 1970); therefore, neither the poorer resolution of DS - CS bands in pigeons compared to standard GAGs, nor the distinctly different overall electrophoretic profiles are surprising (Figures 3 and 4).

A Second Heparitin Sulfate Species

Both SR and WC thoracic GAG electrophoretic profiles were qualitatively identical to SR and WC celiac bifurcation GAG profiles with one exception. At one day and two to three years the WC Celiac bifurcation profiles show a second HS band (HS') (Figures 5 and 6) not found in the WC celiac site of six-week old birds. This HS' band was also found in one of the two analyses of WC celiac GAGs from six-month old birds. No analyses of WC thoracic or of SR celiac or thoracic GAGs showed HS' bands.

Quantitative Analyses

Data is presented as amounts of GAG/mg dry defatted tissue ($\mu\text{g}/\text{mg d.d.wt.}$) and as relative amount of an individual GAG species (percent of total GAG present in any given sample). Comparisons of GAG content in the various breed, aortic site and age combinations are made by examining age trends, breed differences and aorta site differences. Age trends refers to comparisons of GAG content for ages one day, six weeks, six months and two to three years for each of the following breed - site combinations: WC-thoracic; SR-thoracic; WC-celiac; and, SR-celiac aorta sites. Breed differences refer to comparisons of WC vs. SR aortic GAG content for thoracic vs. thoracic at each age and for celiac vs. celiac at each age. Aorta site differences are comparisons of thoracic vs. celiac

Figure 5. Cellulose acetate electrophoresis strips showing WC and SR two- to three-year old celiac foci GAG separations. Arrow indicates position of HS' band, 0 is the origin and 4/6 the Ch 4-SO₄/Ch 6-SO₄ band.

Figure 6. Densitometric tracing of electrophoretically separated celiac foci GAG from two- to three-year old WC. Arrow indicates position of HS' band.

GAG content in WC at each age and in SR at each age.

Total Glycosaminoglycan

Age Trends. Thoracic aortas of WC and SR pigeons contained similar amounts of GAGs at all ages examined (Table 4). The means of all four ages for WC and for SR thoracic GAG content are 21.67 and 20.10 $\mu\text{g}/\text{mg}$ d.d.wt. respectively. Total GAG in the celiac focus nearly doubles in both breeds between one day and six weeks of age. At subsequent ages in SR there is a slight decline, while in WC, total GAG increases between six weeks and six months, but decreases between six months and two to three years of age (Figure 7).

Breed Differences. At all ages examined WC and SR thoracic aorta segments showed similar GAG content but WC celiac segments contained more total GAG than SR celiac segments at corresponding ages. The largest differences in $\mu\text{g}/\text{mg}$ d.d.wt were seen between celiac foci at six-months (7.99) and two to three years (4.06) (Figure 7).

Aorta Site Differences. At all four ages in both breeds the thoracic segments contain more total GAGs than the celiac segments. Differences between one-day old thoracic and celiac GAG in WC and SR are the largest at 14.38 and 14.95 $\mu\text{g}/\text{mg}$ d.d.wt. respectively.

Hyaluronic Acid

Age Trends. In both breeds at both sites, the amount and percent of aorta HA declines between one day and six weeks of age but then increases (Table 5). While WC thoracic aortas show no difference in HA

Table 4. Total Glycosaminoglycans in White Carneau and Show Racer Aortas.

AGE	WC THORACIC	WC CELIAC	SR THORACIC	SR CELIAC
one day	23.35 $\pm$ 2.20 ^a 5 ^b	8.97 1	22.30 $\pm$ 1.82 2	7.35 1
six weeks	20.76 $\pm$ 1.46 7	15.42 1	19.41 $\pm$ 2.34 6	13.03 1
six months	20.54 $\pm$ 2.00 7	19.16 1	18.83 $\pm$ 1.21 7	11.17 $\pm$ 1.21 2
two to three years	22.02 $\pm$ 0.83 5	15.94 1	19.84 $\pm$ 1.05 4	11.88 1

^a Arithmetic means $\pm$ SEM expressed as $\mu\text{g}/\text{mg}$ dry defatted tissue.

^b Number of pools analyzed.

Figure 7. Total Glycosaminoglycan Content of White Carneau (■) and Show Racer (●) Celiac Foci at Four Ages.

between one day and two to three years, WC celiac and SR thoracic and celiac aortic segments have less HA at two to three years than in the embryo.

Breed Differences. Thoracic HA contents in WC and SR are similar at all ages except at two to three years where the thoracic HA content in WC is greater than that of SR. While celiac HA contents ($\mu\text{g}/\text{mg}$ d.d.wt.) in WC and in SR are similar at all corresponding ages, the percent contributed by HA in SR one-day celiac is greater than in WC.

Aorta Site Differences. Comparisons of amounts of the individual GAGs in thoracic vs celiac segments are not meaningful since thoracic aortic segments contain more total GAG than celiac segments in both breeds at all ages. However, comparison of the relative percent HA in thoracic vs. celiac aortic segments shows substantial differences at one day of age in both breeds, with 30-40% of the total GAG in the celiac being HA. However, in WC by two to three years thoracic aorta segments contain more HA than celiac segments.

Heparitin Sulfate

Age Trends. The amount and percent of HS in thoracic aortas of both breeds increases only slightly between one day and six weeks and thereafter remains nearly constant (Table 6). The amount and percent of HS in SR celiac aortic segments increases sharply between one day and six weeks, and then remains approximately constant, while the amount of HS in WC celiac segments also shows a sharp increase between one day and six weeks and subsequently remain nearly constant but the relative percent

Table 5. Hyaluronic Acid in White Carneau and Show Racer Aortas.

AGE	WC THORACIC	WC CELIAC	SR THORACIC	SR CELIAC
one day	2.75 $\pm$.24 ^a (12.1 $\pm$ 1.7) ^b 5 ^c	2.70 (30.1) 1	2.63 $\pm$.43 (12.5 $\pm$ 1.9) 2	2.91 (39.5) 1
six weeks	1.59 $\pm$.20 (7.7 $\pm$.9) 7	0.62 (4.0) 1	0.99 $\pm$.18 (5.0 $\pm$.6) 6	0.64 (4.9) 1
six months	1.64 $\pm$.28 (7.7 $\pm$.9) 7	0.95 $\pm$.22 (5.0) 2	1.62 $\pm$.11 (8.0 $\pm$.6) 7	0.53 $\pm$.31 (5.1 $\pm$ 3.3) 2
two to three years	2.85 $\pm$.15 (13.0 $\pm$.7) 5	1.11 (6.9) 1	1.92 $\pm$.16 (9.7 $\pm$.8) 4	0.85 (7.1) 1

a

Arithmetic means $\pm$ SEM expressed as μ g/mg dry defatted tissue.

b

Relative percents, (HA/total GAG x 100).

c

Number of pools analyzed.

Table 6. Heparitin Sulfate in White Carneau and Show Racer Aortas.

AGE	WC THORACIC	WC CELIAC	SR THORACIC	SR CELIAC
one day	1.60 $\pm$.23 ^a (7.0 $\pm$ 1.4) ^b 5 ^c	2.61 ^d (29.1) 1	2.91 $\pm$ 1.13 (8.4 $\pm$ 3.9) 2	1.68 (22.9) 1
six weeks	2.50 $\pm$.18 (12.4 $\pm$ 1.2) 7	3.92 (25.4) 1	3.40 $\pm$.57 (17.6 $\pm$ 1.8) 6	4.50 (34.5) 1
six months	2.56 $\pm$.13 (13.3 $\pm$ 1.6) 7	3.59 $\pm$.01 ^e (18.7) 2	2.89 $\pm$.22 (14.5 $\pm$ 1.6) 7	3.81 $\pm$.28 (34.3 $\pm$ 1.3) 2
two to three years	2.39 $\pm$.11 (10.8 $\pm$.1) 5	3.96 ^f (24.8) 1	2.58 $\pm$.09 (13.1 $\pm$.9) 4	4.18 (35.2) 1

a

Arithmetic means $\pm$ SEM expressed as $\mu\text{g}/\text{mg}$ dry defatted tissue.

b

Relative percents, (HS/total GAG x 100).

c

Number of Pools Analyzed.

d

Values represent HS + HS' (1.51 μg HS + 1.10 HS'; 16.8%HS + 12.3%HS').

e

One replicate contained only HS (3.60 μg) while the second contained both HS and HS' (2.27 μg HS + 1.30 μg HS'; 12.3%HS + 7.1%HS').

f

Values represent HS + HS' (2.89 μg HS + 1.07 μg HS'; 18.0%HS + 6.8% HS').

HS shows a decrease between one day and six months and an increase by two-to three years of age (Figure 8). When examined separately from total HS the amount of HS in WC celiac segments increases between one day and two to three years of age, while the amount of HS' stays the same. The ratio of HS:HS' therefore is 1.4:1 at one day, 1.7:1 at six months and 2.7:1 at two to three years, thus HS' actually makes up a smaller proportion of WC total celiac HS as a function of age.

Breed Differences. With the exception of one-day celiac segments, SR contain more HS than WC at corresponding sites and ages. In general, differences in percent HS are more striking than differences in amount.

Aorta Site Differences. In either breed, comparisons of percent HS between thoracic and celiac at corresponding ages show that celiac aortic segments contain on an average 2.45 times greater proportion of HS than do thoracic segments.

Dermatan Sulfate

Age Trends. The profile of amounts of DS with age in thoracic aortas of SR shows a small decline between one day and six weeks but thereafter remains approximately the same (Table 7), while the profile of DS in WC thoracic aortas remains nearly constant at all ages. Regardless of breed or age, thoracic DS content is approximately 14%. While celiac profiles of amount of DS with age in either breed show no substantial changes, the relative percent DS at one day is two times that of any other age.

Breed Differences. The amount of DS in WC aortas at either site is nearly identical to that in SR aortas at corresponding sites and ages.

Figure 8. Relative Percents of Heparitin Sulfate (HS/total GAG x 100) in White Carneau (■) and Show Racer (●) Celiac Foci at Four Ages.

Table 7. Dermatan Sulfate in White Carneau and Show Racer Aortas.

AGE	WC THORACIC	WC CELIAC	SR THORACIC	SR CELIAC
one day	3.42 $\pm$.36 ^a (15.1 $\pm$ 2.4) ^b — 5 ^c	2.24 (25.0) 1	3.68 $\pm$.85 (14.2 $\pm$ 4.6) 2	2.06 (28.0) 1
six weeks	2.87 $\pm$.36 (14.0 $\pm$ 1.5) 7	1.79 (11.6) 1	2.34 $\pm$.24 (12.6 $\pm$ 1.5) 6	2.03 (15.6) 1
six months	2.73 $\pm$.40 (13.4 $\pm$ 1.8) 7	1.73 $\pm$.59 (9.0) 2	2.70 $\pm$.30 (13.7 $\pm$ 2.1) 7	1.56 $\pm$.23 (14.4 $\pm$ 3.7) 2
two to three years	3.23 $\pm$.35 (14.8 $\pm$ 2.0) 5	1.89 (11.9) 1	2.71 $\pm$.29 (13.7 $\pm$ 1.3) 4	1.66 (14.0) 1

^a Arithmetic means $\pm$ SEM expressed as $\mu\text{g}/\text{mg}$ dry defatted tissue.

^b Relative percents, (DS/total GAG $\times$ 100).

^c Number of pools analyzed.

Aorta Site Differences. A comparison of percent thoracic DS with celiac DS within either breed shows that only at one age do celiac aorta segments contain a substantially different proportion of DS than thoracic aortas. At one day of age WC and SR celiac foci show 1.7 and 2.0 times the relative percent DS respectively than corresponding thoracic aorta segments.

Chondroitin 4-Sulfate

Age Trends. Age profiles of Ch 4-SO₄ content (amount and percent) were highly variable (Table 8). Both WC thoracic and celiac and SR thoracic aortas show a net decrease in Ch 4-SO₄ content by two to three years of age, while the converse is true for SR celiac Ch 4-SO₄ content. It is not possible to complete the age profile for Ch 4-SO₄ in the celiac site since one-day WC and SR celiac foci sample pools were too small to allow an Elson-Morgan separation of Ch 4-SO₄ from Ch 6-SO₄. Instead, densitometric scanning of cellulose acetate strips was used to quantitate total Ch 4/6-SO₄ present (Table 9).

Breed Differences. A comparison of WC thoracic and SR thoracic Ch 4-SO₄ contents at corresponding ages shows no appreciable differences. However in WC celiac vs. SR celiac comparisons, WC contain larger quantities of Ch 4-SO₄ (both $\mu\text{g}/\text{mg}$ d.d.wt. and percent) than SR at six weeks and at six months, while the converse is true at two to three years of age (Figure 9).

Aorta Site Differences. Comparisons of percent Ch4-SO₄ between thoracic and celiac sites within either breed at corresponding ages were variable. White Carneau thoracic aortas contain approximately the same amounts of Ch4-SO₄ as do celiac aortic segments at six-weeks and at two-

Table 8. Chondroitin 4-Sulfate in White Carneau and Show Racer Aortas.

AGE	WC THORACIC	WC CELIAC	SR THORACIC	SR CELIAC
one day	3.82±.92 ^a (15.2±2.1) ^b 5 ^c	— ^d	2.79±.66 (12.4±2.0) 2	— ^d
six weeks	3.54±.61 (16.8±2.2) 7	2.63 (17.1) 1	3.94±.63 (18.0±1.5) 6	0.81 (6.3) 1
six months	3.48±.62 (16.1±1.7) 7	4.23 (22.1) 1	3.83±1.05 (17.4±4.0) 7	0.74±.10 (6.6±.4) 2
two to three years	2.30±.42 (10.4±1.8) 5	1.51 (9.5) 1	1.85±.45 (9.3±2.1) 4	2.21 (18.6) 1

^a Arithmetic means ± SEM expressed as µg/mg dry defatted tissue.

^b Relative percents, (Ch 4-SO₄/total GAG × 100).

^c Number of pools analyzed.

^d Values reported in Table 9 as Ch 4-SO₄ + Ch 6-SO₄.

Figure 9. Amounts of Chondroitin 4-Sulfate in White Carneau (■) and Show Racer (●) Celiac Foci at Three Ages.

to three years but celiac segments contain more Ch4-SO_4 than thoracic segments at six months of age. Show Racer thoracic aortas contained larger relative amounts of Ch4-SO_4 at six weeks and six months (2.9 and 2.6 x respectively) than celiac sites, while at two to three years the converse was true.

Chondroitin 6-Sulfate

Age Trends. Profiles of Ch6-SO_4 content within the thoracic aorta of either WC or SR show little change with age, and Ch6-SO_4 is present in large amounts compared to the other GAGs, constituting roughly 50% of total thoracic GAG in both breeds (Table 9). The WC celiac Ch6-SO_4 profile shows a decisive increase between one day and six weeks but remains approximately constant at subsequent ages. The SR celiac profile also shows a marked increase between one day and six weeks and then remains nearly constant until six months when it declines slightly (Table 9 and Figure 10).

Breed Differences. While WC thoracic and SR thoracic aortas at corresponding ages show very similar amounts and percents of Ch6-SO_4 , WC celiac Ch6-SO_4 content is consistently higher than that of corresponding SR at all four ages, particularly at two to three years (2.5 x amount, 1.9 x %) (Table 9 and Figure 10).

Aorta Site Differences. The relative percents of Ch6-SO_4 are nearly equal between WC thoracic and celiac segments at all ages other than one day where the thoracic proportion of Ch6-SO_4 is 3.4 times that of the celiac. (The WC and the SR celiac data presented for Ch6-SO_4 in embryos in Table 9 actually represents a mixture of Ch4-SO_4 and Ch6-SO_4 isomers).

Table 9. Chondroitin 6-Sulfate in White Carneau and Show Racer Aortas.

AGE	WC THORACIC	WC CELIAC	SR THORACIC	SR CELIAC
one day	12.49 $\pm$ 1.35 ^a (53.2 $\pm$ 3.4) ^b 5 ^c	1.42 ^d (15.8) 1	11.48 $\pm$ 2.14 (52.5 $\pm$ 12.4) 2	0.70 ^d (9.6) 1
six weeks	10.27 $\pm$ 1.13 (49.0 $\pm$ 3.4) 7	6.47 (42.0) 1	9.94 $\pm$ 1.12 (51.9 $\pm$ 2.7) 6	5.04 (38.7) 1
six months	10.18 $\pm$ 1.22 (49.4 $\pm$ 3.1) 7	8.66 $\pm$.10 (45.2) 2	9.55 $\pm$.57 (46.4 $\pm$ 1.3) 7	4.53 $\pm$ 1.36 (39.7 $\pm$ 7.9) 2
two to three year	11.24 $\pm$.69 (51.0 $\pm$ 2.3) 5	7.47 (46.9) 1	10.79 $\pm$.88 (54.2 $\pm$ 2.0) 4	2.99 (25.1) 1

^a Arithmetic means $\pm$ SEM expressed as $\mu\text{g}/\text{mg}$ dry defatted tissue.

^b Relative percents, (Ch 6-SO₄/total GAG x 100).

^c Number of pools analyzed.

^d These Ch 6-SO₄ values represent Ch 4-SO₄ and Ch 6-SO₄ combined.

Figure 10. Amounts of Chondroitin 6-Sulfate in White Carneau (■) and Show Racer (●) Celiac Foci at Four Ages. (One-day Ch 4-SO₄ values represent Ch 4-SO₄ and Ch 6-SO₄ combined).

A comparison of the relative percent Ch6-SO_4 between SR thoracic and celiac aorta segments shows nearly equal proportions of Ch6-SO_4 at six months of age but larger relative quantities of Ch6-SO_4 in thoracic at one-day (5.5 x), six weeks (1.3 x) and two to three years (2.2 x).

DISCUSSION

Qualitative Analysis

Pigeon and Standard Glycosaminoglycan Electrophoretic Species

Since the structure of a GAG may vary, depending upon species and tissue of origin, it was not surprising that pigeon aorta HS and CS did not co-electrophorese with standard HS and CS respectively (Figures 3 and 4). Heparitin sulfate from pigeon thoracic aorta, like that isolated from human aorta (Cifonelli and King, 1970) had a relatively slow electrophoretic mobility compared to the beef lung HS used as standard. The slower mobility of pigeon aorta HS may be due to the presence of non-sulfated disaccharide repeating units within the molecule, to a low N-SO₄:O-SO₄ ratio, or to fewer L-iduronic acid-containing disaccharides.

The slower mobility of pigeon CS relative to standard CS may be the result of several factors. The standard CS band was a 50:50 mixture of whale cartilage Ch4-SO₄ and shark cartilage Ch6-SO₄, and also had a different Ch4-SO₄:Ch6-SO₄ ratio than pigeon aorta CS. (Most pigeon CS bands were predominantly Ch6-SO₄). Some heterogeneity in site and extent of sulfation is likely in pigeon CS isomers, as well as in the presence of L-iduronic acid-N-acetyl galactosamine-sulfate disaccharides in addition to normal D-glucuronic acid-N-acetyl galactosamine-sulfate disaccharides. Hybrid copolymers of CS-DS such as those found by Malmstrom et al. (1975) may also account for the slow migration of pigeon CS and the poor resolution of DS-CS bands relative to standards. The fact that one-day old pigeon GAG electrophoretic profiles show the poorest DS-CS

resolution may be indicative of lack of biosynthetic specificity at this early stage in development. By six weeks of age the separation of DS from CS improves, suggesting less heterogeneity in the GAGs synthesized at this age. No further changes occur in electrophoretic profiles after six weeks of age, perhaps because the maturation of GAG biosynthetic pathways in pigeon thoracic aorta is complete by this age. It is interesting to note that pigeon thoracic aortas have reached almost full size by six weeks of age.

A Second Heparitin Sulfate Species

The unique presence of a second type of HS, HS', in the celiac bifurcations of WC pigeons (not in WC thoracic aortas or in SR at either site) may be of significance. Since the celiac focus is the predisposed site of atherosclerotic lesion formation, and since WC are more prone to developing lesions than are SR, the presence of HS' in the celiac foci of WC could play a role in atherogenesis. Heparitin sulfate' has a higher electrophoretic mobility than its normal HS counterpart (Figures 5 and 6), a characteristic which could be due to a higher sulfate content or to the presence of L-iduronic acid moieties. If HS' is an oversulfated form of HS, the position of these additional sulfate groups ($O-SO_3$ or $N-SO_3$) would influence its physiological properties. An increase in $N-SO_3$ in HS' would result in increased lipoprotein lipase activating effects and anticoagulant activity (Grossman and Cifonelli, 1962) by this species, both of which would retard atherogenesis. Conversely, the presence of additional sulfate groups in HS' may promote atherogenesis since the negative

charge of the GAGs is responsible for promoting GAG-LDL and-VLDL interactions (Berenson et al. 1971; Amenta and Waters, 1960; Iverius, 1972 and others). Srinivasan et al. (1970) showed that HS had the highest affinity for VLDL and LDL of all GAGs tested, and suggested that this was due specifically to the N-SO₄ content of HS (compared to the O-SO₄ found in other aortic GAGs). If HS plays a role in cell division (Kræmer and Tobey, 1972), then the presence of a variant HS' at the surface of WC celiac smooth muscle cells may facilitate the focal proliferation of smooth muscle cells which is a prerequisite for lesion formation at this site. This idea is supported by the hypothesis of Benditt and Benditt (1973) which states that the smooth muscle cells found in atherosclerotic lesions are actually transformed cell populations, and transformed cells synthesize variant forms of HS (Sato et al., 1973 and Sato et al., 1974).

Why HS' was detected at one day of age, at two to three years, and in one of two six-month celiac pools and not at six weeks of age is difficult to explain. The small size of celiac segments made analyses of several replicate pools impossible, and furthermore an HS' profile with age in individual birds could not be determined. However, if HS' is involved in atherogenesis then its presence as early as one-day of age at the celiac site implies a genetic predisposition to atherosclerosis in WC pigeons, and an undetermined role for HS' in early atherogenesis.

It is possible that some individual celiac segments contain only HS or HS' rather than a mixture of the two, but since 1-day old pools represent more than three hundred segments and each pool of older age groups, more than twenty-five segments, analysis of individual celiac segments to test this hypothesis is obviously impossible with available methodology.

Quantitative Analyses

In previous studies which have employed the model system of Santerre et al. (1972) to study changes in properties or composition of the blood vessel wall occurring as a function of age or atherogenesis, the following types of comparisons were made: WC thoracic vs. SR thoracic; thoracic vs. celiac within each breed and WC vs. SR celiac. Lipid and collagen contents, enzyme activities and respiratory control ratios in SR and WC thoracic aortas were comparable at various ages and were considered representative of the normal aging process in both breeds (Nicolosi, Santerre and Smith, 1972; Santerre, Nicolosi and Smith, 1974; Subbiah, Kottke and Carlo, 1974 and St. Clair, Toma and Lofland, 1975). Comparisons between thoracic and celiac sites in WC at various ages, however showed differences. Age profiles of the thoracic segments were considered characteristic of normal aging, while the changes in celiac segments were considered characteristic of atherosclerotic degeneration. Similar comparisons between thoracic and celiac sites in SR up to one year of age also showed differences due to aging and atherogenesis, but after one year SR celiac sites degenerate at a much slower rate than WC and further changes at this site are much less evident (Santerre et al., 1972). (In comparable studies of aortic GAG in species other than pigeons, changes in GAG profiles of uninvolved and involved segments were also characteristic of aging and lesion progression respectively [Velican, 1974; Kumar et al., 1967a and Kumar et al., 1967b]).

Finally, the most pronounced differences were found in comparisons made between WC and SR celiac segments and were considered indicative of the different susceptibilities of these breeds to atherosclerosis (Nicolosi et al., 1972; Santerre et al., 1974; Subbiah, et al., 1974 and St. Clair et al., 1975).

In the discussion which follows, GAG profiles with age in thoracic segments are considered characteristic of the normal aging process, while those in celiac segments of WC are considered characteristic of atherogenesis.

Total Glycosaminoglycans

Age Trends. In agreement with the work of Kumar et al. (1967a) and Schmidt and Dmochowski (1964a), no appreciable changes in total GAG content of thoracic aortas with age (Table 4) were found. White Carneau celiac segments however, showed progressive increases in total GAG by six-weeks and six months of age (Figure 7). This increase in aortic GAG prior to the onset of fatty streaks is characteristic of the atherosclerotic disease process (Velican, 1974; Kumar et al., 1967b; Wagh et al., 1973 and Hollander et al., 1968). The decline in aortic GAG content in WC celiac segments by three years of age agrees with the previous work of Bottcher and Klynstra (1962) which showed a decrease in aortic GAG as the lesion developed past the fatty streak stage of the disease. (Most three-year old WC pigeons possess well developed macroscopic celiac lesions while three-year old SR exhibit virtually no lesions). It is interesting to note that while SR celiac segments show an increase in GAG content between one day and six weeks of age this value declines by six months of

age. The absence of further increases in GAG in SR celiac segments may be partially responsible for the resistance of this breed to atherosclerosis (Figure 7).

Santerre et al. (1972) showed little difference between the two breeds up to one year of age in incidence, location or histopathology of atherosclerotic lesions. After one year however, SR had a much lower lesion severity index than WC at the celiac bifurcation.

Breed Differences. Absence of differences between WC and SR thoracic aorta GAG content at all ages examined was expected, since neither breed develops lesions in the thoracic segment. Celiac segments of WC however, particularly at six months and two to three years of age contained larger quantities of GAG than SR, thus supporting the correlation between amount of aortic GAG and species susceptibility reported by previous workers (Robinson et al., 1975; Gero et al., 1973; Mullinger and Manley, 1969 and Gardias et al., 1973) (Figure 7).

Aorta Site Differences. Despite their higher GAG content thoracic aortas do not develop lesions, which implies that factors other than merely quantity of GAG play a part in the atherogenic process occurring at the celiac foci. Thoracic and celiac segments are different in organization, the later being more muscular and the former more elastic in nature. Nanov et al. (1974); Souadjian et al. (1969) and Stefanovich and Akiyama (1970) not only reported larger quantities of GAG in elastic aortas than in muscular aortas, but also found lesions in distal but not proximal portions of the aorta (proximal portions of the aorta are more elastic than distal portions).

Haemodynamic irregularities and anoxia may cause local metabolic disturbances at the celiac site. Smooth muscle cells within the cushion could then respond by elaborating types of GAGs qualitatively distinct from those present in thoracic aorta. (This is certainly the case in the present study with reference to HS' and the percents of total HS and C h6-SO₄ found in celiac segments.) If the cells of the thoracic and celiac segments possess different extracellular environments, then extracellular GAGs could in turn affect the metabolic capacities for growth, repair and remodeling in these two sites differently. Furthermore GAGs in the extracellular space which bind lipid may present an additional challenge to celiac cells because of the unique arterial structure (architecture) at this site (described on page 84).

Hyaluronic Acid

Age Trends. The decrease in HA content in SR thoracic aortas between one-day and two to three years (Table 5) of age agrees with the majority of reports, which show decreases in aortic HA content with age (Meyer, 1969 and Muir, 1964). The decrease and subsequent increase in WC thoracic HA content is atypical but similar to the findings of Vijayakumar et al. (1975). The relatively high proportion of HA in celiac foci at one day of age (>30%) found in both breeds is unusual compared to previous studies (Schmidt and Dmochowski, 1964a; Nakamura et al., 1971 and Klynstra et al., 1967) which showed only about 10% aortic HA content. However, since these studies did not analyze predisposed branch points within the thoracic aorta separately, any large focal concentrations of HA may have been obscured. The large celiac content of HA at one-day of age is consis-

tent with the idea that large quantities of HA are found in developing and remodeling tissues (Toole, 1973 and Muir, 1964). The subsequent decrease in celiac HA content agrees with previous reports of declining HA content during atherogenesis (Schmidt and Dmochowski, 1964a; Nakamura et al., 1971 and Klynstra et al., 1967).

Breed Differences. The larger HA content in WC thoracic aortas compared to SR thoracic aortas at two to three years may reflect differences in turgor, water content, and viscoelasticity between breeds at this age. The difference (.93 $\mu\text{g}/\text{mg}$ d.d.wt.) is small however, and probably does not have great biological significance. The similar celiac HA content between breeds would imply that HA does not play a significant role in atherogenesis. This hypothesis is supported by studies which show very weak associations between HA and plasma LP if they interact at all (Srinivasan et al., 1970 and Iverius, 1972). The excluded volume effect, and molecular sieving of HA proposed by Velican (1974) are probably slight since older celiac segments contain only about 6% of total GAG as HA, and LP accumulate at increasingly greater rates as the fatty streak develops.

Aorta Site Differences. The larger proportion of HA in WC and in SR celiac segments at one day when compared to corresponding thoracic segments may indicate that the celiac segments are undergoing more extensive changes than the thoracic segments at this age. These celiac-thoracic differences disappear by six weeks of age, and only in WC at two to three years does a difference in celiac vs. thoracic HA content reappear. Here, the smaller relative percent HA in the celiac site may cause a loss in turgor and viscoelasticity in WC celiac compared to WC

thoracic sites.

Heparitin Sulfate

Age Trends. Since the profile of total HS with age (Table 6) in both breeds follows the same trends in both thoracic and celiac sites (increase between one day and six weeks but constant at subsequent ages) this trend is probably part of a normal aging or maturation process, and is without significance in the atherogenic process.

The implications of increased HS:HS' ratios with age in WC celiac foci remain obscure. If atherosclerosis resistant species do, indeed contain relatively more HS than susceptible species (Gardias et al., 1973 and Mullinger and Manley, 1969) then the elaboration of HS' by WC may represent an effort to prevent and retard lesion formation. The variant nature of HS' however, may accelerate those very processes if the affinity of HS' for plasma LP outweighs its anticoagulant and lipoprotein lipase-activating effects.

Breed Differences. Although SR thoracic aortas consistently contain more HS than WC thoracic aortas at corresponding ages these differences are of such small magnitude (average 2.5% for all four ages) that they are probably without major significance. However, celiac segments of SR at six weeks, six months and two to three years contain an average of 11.7% higher HS content than WC celiac segments even when HS' is included in the WC HS values (Figure 8).

This finding is in agreement with reports of Gardias et al. (1973); Hermelin et al. (1974) and Mullinger and Manley (1969) which showed higher

proportions of HS in atherosclerosis-resistant species than in susceptible species. The lipoprotein lipase activating effects and anticoagulant properties of HS (Cifonelli and King, 1970) would seem to overbalance its reputed affinity for LP (Srinivasan, 1970) in the atherosclerosis resistant SR pigeon. Again, it may be that the type of HS (HS or HS') is more important than the relative amounts of HS present in determining the physiological properties it imparts to the blood vessel wall.

Aorta Site Differences. The larger relative HS content found in celiac aortic segments of both breeds when compared with thoracic segments agrees with previous studies which showed a much greater proportion of HS in abdominal aortas than in thoracic aortas (Farquhar and Dunstone, 1968; Stefanovich and Akiyama, 1970 and Engel, 1971). (Celiac foci are more representative of muscular abdominal aorta than of elastic thoracic aorta).

While the higher HS content in SR celiac when compared to WC celiac segments is in agreement with previous reports of higher HS content in atherosclerosis-resistant species (Gardias et al., 1973; Hermelin et al., 1974 and Mullinger and Manley, 1969), the higher HS content in celiac segments (in both breeds) when compared to thoracic segments complicates interpretations of these results. If a high proportion of HS in aortic tissue enhances atherosclerosis resistance, then some additional factors must play a part in initiation and development of lesions at the bifurcation since celiac but not thoracic segments develop lesions. The elaboration of HS by smooth muscle cells at the celiac site may represent an effort to retard atherogenesis, one which is not successful due to local

haemodynamic stress, anoxia, lipid infiltration or nutritional, metabolic or transport inadequacies, etc.

Dermatan Sulfate

Age Trends. Although SR thoracic aortas at one day showed a larger amount of DS (approximately $1.0 \mu\text{g}/\text{mg}$ d.d.wt. higher) than at subsequent ages, the low number of replicates (2) and large SEM ($\pm .85$) for the value reported (Table 7) permit only preliminary interpretation of this data. Thoracic aortas of both breeds showed similar DS contents (as percent) at all ages examined, in agreement with the work of Nakamura et al. (1971). While celiac segments of both breeds also contain similar proportions of DS at corresponding ages, the relative percent DS at one day is higher than at subsequent ages. This drop in relative percent DS by six weeks of age in celiac sites of both breeds as well as in SR thoracic sites, occurs simultaneously with the increase in relative percent of HS, and probably reflects the change in aortic HS composition and is not a primary effect.

Breed Differences. Since there are no appreciable differences between breeds in absolute or relative quantities of DS present at thoracic or celiac sites it would seem that DS does not play an important role in atherogenesis. Any of the "protective" anticoagulant, antithrombic and lipid-clearing capacities which DS is reported to display in the blood vessel wall (Velican, 1971 and Dorfman, 1963), must be exhibited in aortas of both breeds to similar extents. Furthermore, if DS aids in the extra-cellular organization of collagen (Obrink, 1973), or traps plasma LP

(Iverius,1972) these effects must also be similar in both WC and SR aortas.

Aorta Site Differences. The larger relative quantities of DS at one day of age in both WC and SR celiac segments may be indicative of greater collagen biosynthesis in the celiac foci compared to the thoracic aorta at this age. Gan et al.(1967) have reported that the muscular abdominal segments of chicken aorta contained more collagen than the elastic thoracic aorta (the celiac site is muscular in nature). St. Clair et al. (1975) reported significantly more collagen in plaques of WC pigeons with naturally-occurring atherosclerosis than in normal tissue from the same aorta. Alternatively, high DS may simply reflect low relative HS content in one-day celiac segments.

Chondroitin 4-Sulfate

Age Trends. The net decrease in amount and percent of aorta Ch4-SO_4 between one day and two to three years seen in both breeds is of small magnitude and is probably the result of the normal aging process in pigeon thoracic aortas (Table 7). However, the age profiles of Ch4-SO_4 in WC celiac segments were opposite in SR (Figure 9). Since the majority of properties reported for Ch4-SO_4 are antiatherogenic in nature (Morrison and Schjeide,1974 and Nakashima et al.,1975), a decreased level of Ch4-SO_4 at two years of age may be related to susceptibility to atherosclerosis in WC celiac foci. Only advanced lesions develop thromboses of fibrinogen and platelets (Watts,1971; Walton and Williamson,1968 and Morrison and Schjeide, 1974) and protective, antithrombogenic and antiatherogenic effects of Ch4-SO_4 appear to be lost in WC prior to the onset of severe or

complicated lesions. It is interesting to note that the grade of celiac lesions in SR and WC are similar until after one year of age when the severity index increases in WC but plateaus in SR (Santerre et al. 1972). These differences in lesion scores with age between WC and SR are manifest at the same time when Ch4-SO₄ content in SR celiac sites first exceeds that in WC celiac sites (Figure 9). It may be that Ch4-SO₄ content does not inhibit early atherogenesis, but rather retards subsequent lesion progression.

Breed Differences. The similar Ch4-SO₄ content in thoracic aortas of both breeds at corresponding ages is consistent with the idea that changes in GAG content in pigeon thoracic aortas are representative of aging and not atherogenesis. The tendency for WC celiac sites to contain less Ch4-SO₄ than SR celiac sites by two-to three-years of age may have pathological significance. Chondroitin 4-sulfate is reputed to possess antiatherogenic, antithrombogenic and lipid-clearing activities and to prevent swelling of LDL which aids in their deposition within the blood vessel wall (Morrison and Enrick, 1973; Morrison and Schjeide, 1974 and Nakashima et al., 1975). A higher level of Ch4-SO₄ in SR celiac sites (18.6%) compared to WC celiac sites (9.5%) may partially explain SR resistance to advanced atherosclerosis if the higher Ch4-SO₄ content, or Ch4-SO₄:Ch6-SO₄ ratios in older SR aids in lipid clearing and prevents thrombus formation and lesion progression (Figure 11).

Aorta Site Differences. The implications of comparisons made concerning Ch4-SO₄ contents in thoracic and celiac sites remain obscure. If Ch4-SO₄ is protective or antiatherogenic in nature, then the similar Ch4-SO₄ contents in thoracic and celiac sites at corresponding ages

Figure 11. The Ratio of Chondroitin 4-Sulfate:Chondroitin 6-Sulfate in White Carneau (■) and Show Racer (●) Celiac Foci at Three Ages.

(with the exception of two-to three-years old SR) must safeguard both sites to a similar extent. (At two to three years SR celiac sites contain two times the relative amount of Ch4-SO₄ in SR thoracic sites).

Chondroitin 6-Sulfate

Age Trends. In agreement with the work of Velican (1974) and Murata (1974), Ch6-SO₄ is the major component of the thoracic aorta, constituting 51% of total GAG (Table 8). In addition, the proportions of DS:Ch4-SO₄:Ch6-SO₄ found in the present study (1:1:3.6) were similar to those reported by Murata (1974) (1:1:2.5). Both WC and SR thoracic aortas showed no changes in Ch6-SO₄ content with age, in agreement with the report of Kumar et al. (1967a) on human thoracic Ch6-SO₄ age profiles.

The increases in the Ch6-SO₄ content between one day and six weeks of age in celiac sites in both breeds (Figure 10) may represent increased collagen synthesis (Muir, 1969) and decreased permeability (Klynstra, 1974 and Bottcher and Klynstra, 1965) during this time period. As the Ch6-SO₄ content increased in the extracellular matrix, a more entangled network of GAGs would form. Plasma components which were propelled into this matrix would be retarded and would eventually accumulate. Only SR celiac shows a subsequent decrease in Ch6-SO₄ content (Figure 10) which should lead to an increase in permeability by two to three years of age and lessen the molecular sieving of plasma LP within this portion of the blood vessel wall. Again, the decline in Ch6-SO₄ content after six months of age in SR coincides with a leveling off of lesion severity in this breed, a phenomenon which does not occur in WC.

Breed Differences. The similar Ch6-SO₄ content of both SR and WC thoracic sites fits the common trends that neither breed develops thoracic lesions and that GAG aging patterns are common to both breeds at this site. By six weeks of age Ch6-SO₄ is the major GAG present in WC celiac foci, in agreement with previous studies which showed that Ch 6-SO₄ is the major GAG found in aortas of atherosclerosis-susceptible species (Gardias et al., 1973; Hermelin et al., 1974 and Mullinger and Manley, 1969). In atherosclerosis-resistant SR celiac foci, however HS content approximates or exceeds Ch6-SO₄ content at the ages examined (Tables 6 and 9), while in susceptible WC celiac foci this is true only at one day of age. An examination of the ratios of Ch6-SO₄:HS in celiac foci shows consistently larger values in WC vs. SR at one day (.54 vs. .42), six weeks (1.65 vs. 1.12), six months (2.41 vs. 1.19) and two to three years of age (1.89 vs. .72). The larger amounts and percents of Ch6-SO₄ in WC celiac foci when compared with SR foci at corresponding ages (particularly at two to three years) may cause increased seiving and retention of plasma LP (Bottcher and Klynstra, 1965; Klynstra, 1974 and Srinivasan et al., 1970), and inhibition of β -glucuronidase and hyaluronidase (Velican, 1971) in WC at this site. All of these effects of Ch6-SO₄ would enhance lesion formation in WC celiac bifurcations by increasing lipid deposition and interfering with the degradation and turnover of matrix GAGs. In the genetic mucopolysaccharidoses, disturbances in normal lysosomal catabolism of GAGs lead not only to the accumulation of GAG but to lipid accumulation as well (Hers, 1973; Neufeld and Cantz, 1973 and Muir, 1969). Apparently accumulation of GAGs within the lysosome also interferes with normal turn-

over of complex lipids, causing massive deposits of lipids. In advanced lesions in WC, smooth muscle cells are actually filled with lipid vacuoles, some of which resemble congested lysosomes (Cooke and Smith, 1968 and Wight, 1972). Not only would defective GAG catabolism prevent degradation of endocytosed ground substance GAG but the missing or defective enzymes may prevent crinophagy as well. (Crinophagy refers to lysosomal digestion of secretory products formed in excess of needs and accomplishes an important regulatory function [Hers, 1973]). If crinophagy was retarded or absent in WC celiac smooth muscle cells then more GAG would be secreted into the extracellular space than at SR celiac bifurcations.

Aorta Site Differences. The proportions of Ch 6-SO₄ in WC thoracic aorta segments at six weeks, six months and two to three years and in SR thoracic segments at six months were of about the same proportion as in corresponding celiac segments, paralleling previous findings of similar relative Ch 6-SO₄ contents in thoracic and abdominal aortas (Farquhar and Dunstone, 1968; Stefanovich and Akiyama, 1970 and Engel, 1971). The celiac focus is the predisposed site for lesion formation, and the presence of factors which cause LP accumulations or decrease permeability and enzyme activities (ie. high Ch 6-SO₄ content) could promote atherogenesis. Thus the smaller proportions of Ch 6-SO₄ found in SR celiac at one-day, six weeks and two to three years when compared to corresponding thoracic sites may lessen the tendency for atherogenesis at the celiac bifurcation in this breed. Any beneficial effects of low Ch 6-SO₄ in celiac sites of WC are lost before six weeks of age.

Conclusions

The present study may in part explain susceptibility and resistance of the two breeds of pigeons to atherosclerosis, by describing changes in ground substance GAGs which occur during aging and atherogenesis, and by relating various GAGs to the pathogenic sequence.

The larger quantities of GAGs found in celiac foci of WC when compared to SR foci correlate with breed differences in susceptibility to atherosclerosis, since the reported affinities of GAGs in the blood vessel wall for plasma LP would explain the deposition of more lipid in the GAG-rich arterial walls of WC. Previous studies by Nicolosi et al. (1972) and Subbiah et al. (1974) showed more total lipid, and in particular more cholesteryl esters and squalene, in six-month WC celiac sites when compared with SR celiac sites. In order to know whether GAG changes precede differences in lipid content of WC and SR celiac foci, lipid analyses must be performed on one-day and six-week old birds as well.

The type of GAGs present in celiac foci of these two breeds, as well as overall quantities, may also be of pathogenic significance. The larger proportion of HS (from age 6 weeks and later) and lack of HS' in SR may aid in their resistance to atherogenesis. The idea of HS preventing or retarding atherogenesis in some way (due to anticoagulant and lipoprotein lipase activating effects?) is based on the previous findings of higher HS levels in atherosclerosis-resistant species (Gardias et al., 1973; Hermelin et al., 1974 and Mullinger and Manley, 1969). The nature of the properties of HS and, in particular, HS' in the blood vessel wall must be elucidated by studying the distributions (cell surface or matrix),

carbohydrate and sulfate contents, and the abilities of HS and HS' to interact with plasma LP before a more definitive hypothesis concerning HS involvement in atherogenesis can be made. The consistently larger Ch6-SO₄ content in WC celiac foci may be of particular importance in electrostatically binding LP and also in retaining LP propelled into the matrix by molecular sieving effects. The decreasing amounts of Ch4-SO₄ in WC aortas after six months of age may contribute to lesion progression if these smaller amounts indicate lessening of antithrombic, lipolytic and LP "antischwelling" effects at the celiac bifurcation. The ratios of Ch4-SO₄ to Ch6-SO₄ present in celiac foci would appear more important than the absolute amounts of these two GAGs since their reputed effects on lesion development are antagonistic (Ch4-SO₄ being "anti-atherogenic" and Ch6-SO₄, "atherogenic").

The absence of differences in thoracic GAG profiles between WC and SR is suggestive of similar metabolic and aging processes in the non-predisposed or lesion-free sections of the thoracic aorta in both breeds. In general, the changes in aortic GAG patterns and contents which occurred during aging in WC and SR thoracic segments were of smaller magnitude and were less variable than the changes occurring in the celiac segments of these birds.

If the primary role of GAGs in atherogenesis is to enhance LP deposition within the blood vessel wall, then further consideration must be given to the finding that the relatively GAG-poor celiac foci and not the GAG-rich thoracic aortas develop atherosclerotic lesions. The presence of a raised muscular cushion represents a normal feature of vascular architecture at the celiac bifurcation. This aggregation of smooth muscle

cells is a necessary prerequisite but not a sufficient condition for atherogenesis (Cooke and Smith, 1968 and Nicolosi et al., 1972). The cells in the cushion display different characteristics than those in the thoracic trunk; they are smaller, more fusiform, and are not organized into the laminae characteristic of thoracic smooth muscle cells. Furthermore, elastic laminae are not as abundant, and celiac cells are packed closer together, allowing less room for matrix components than in thoracic segments (Cooke and Smith, 1968 and Lauper et al., 1975). Probably, the unique 3-dimensional architecture of the extracellular space at the celiac focus, as well as the altered structures and proportions of GAGs contributes to lipid accumulations here. Thus design, as well as composition, of celiac ground substance may form what Morrison referred to as "unsound matrix" (Morrison and Schjeide, 1974).

The types and amounts of matrix GAG which celiac smooth muscle cells elaborate may be different than those elaborated by thoracic cells because of the basic differences in elasticity and muscularity of these two segments and because of the natures of the stresses placed upon these two sites. Various haemodynamic, hypoxic, toxic and hormonal factors which affect the celiac bifurcation may be responsible for focal alterations in GAGs at this site. The elaboration of GAGs in distinctive patterns at the celiac site may initially represent a nonspecific response to injury expressed by both breeds, a response which causes lipid deposition. This idea is supported by previous work (Santerre, et al., 1972) which showed almost identical alterations in WC and SR celiac foci up until one year of age. White Carneau foci, however, undergo additional degeneration to a diseased state which is not expressed in SR

until a much later age. The increase in Ch6-SO_4 and subsequent decreases in HS, Ch4-SO_4 and the ratio of $\text{Ch4-SO}_4:\text{Ch6-SO}_4$ shown in WC but not in SR celiac foci occurs just prior to the manifestation of different lesion progression patterns in these two breeds of pigeons. Goodman and Herndon (1963) suggested that atherosclerosis in pigeons resulted from an alteration of some metabolic factor which is genetically controlled, and the genetic factors responsible for initiation of microscopic lesions are largely independent of those responsible for the progress of lesions to gross plaques. The pronounced differences in GAG content between WC and SR celiac foci after six months of age, (in contrast to more similar breed patterns at earlier ages) may represent alterations in the metabolic capacities of WC smooth muscle cells to synthesize and to degrade GAGs, alterations which may be responsible for lesion progression in WC.

If GAG matrix components play a role in the initiation of microscopic lesions, the role must be similar in WC and SR since differences in GAG content appear after atherogenesis has begun. The only distinctive feature of WC celiac foci at one day of age is the presence of HS'. This unique HS species may influence the proportions and total amount of GAGs elaborated in older WC celiac foci. In addition, WC and SR smooth muscle cells may elaborate different GAG matrix components after six-months of age because they react to the similar stresses placed upon them in different ways. Zemlenyi and Rosenstein (1975) suggested that metabolic differences between these two pigeon strains are of an "inherited or genetic nature" and Goodman and Herndon (1963) suggested a polygenic system as the most probable genetic mechanism predisposing WC to atherosclerosis.

Regardless of the exact nature of the factors involved, WC celiac foci elaborate different types and amounts of GAGs than corresponding SR foci, and according to the extracellular matrix hypothesis of Hay and Meier (1974), once present, these different GAG patterns in WC and SR celiac ground substance would influence metabolic pathways in these two breeds differently. For example, WC may be less able to metabolize LP, to dissolve fibrin clots, or may have more congested lysosomes than SR. The structure and composition of GAGs at the celiac bifurcation may be genetically determined or may be affected by cushion architecture and various factors or stresses operating at this site. Distinctive focal GAG patterns in blood vessel walls, particularly at branch points, could be the prerequisites that lead to the secondary accumulations of lipids, fibrin, calcium, and other materials in arterial lesions. Since the genesis of atherosclerosis is complex, multiple factors must contribute to aortic degeneration. Alterations in GAG of the ground substance may occur simultaneously with, or be followed by, modification of collagen and elastin fibers. These alterations in ground substance components may together influence the functions and properties of the celiac foci, disposing them to atherosclerosis.

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APPENDIX

Table 1. Accuracy Determination for Hyaluronic Acid.

<u>Experiment Number</u>	<u>Number of Points Per Line</u>	<u>Predicted Value Unknown - $\mu\text{g}/\mu\text{l}$</u>	<u>Actual Value Unknown - $\mu\text{g}/\mu\text{l}$</u>	<u>Residual Error- $\mu\text{g}/\mu\text{l}$</u>
1	7	0.20	0.17	0.03
2	6	0.75	0.77	0.02
3	6	1.00	0.97	0.03
4	4	0.38	0.38	0.00
5	5	0.25	0.26	0.01
6	5	1.50	1.46	0.04
7	4	0.50	0.52	0.02
8	4	0.38	0.41	0.03
9	5	0.25	0.24	0.01
10	3	0.75	0.75	0.00
11	4	0.20	0.21	0.01
12	4	0.50	0.50	0.00
13	4	0.38	0.33	0.05
14	5	0.25	0.24	0.01
15	4	0.20	0.19	0.01
16	4	0.75	0.76	0.01
17	4	0.50	0.48	0.02
18	4	0.38	0.38	0.00
19	5	1.00	1.01	0.01
20	4	0.20	0.20	0.00
21	5	0.75	0.77	0.02
22	4	0.50	0.50	0.00
23	3	1.00	1.01	0.01

Table 1. (Cont'd)

24	4	0.25	0.23	0.02
25	5	0.38	0.37	0.01

Table 2. Accuracy Determinations for Heparitin Sulfate.

<u>Experiment Number</u>	<u>Number of Points Per Line</u>	<u>Predicted Value Unknown - $\mu\text{g}/\mu\text{l}$</u>	<u>Actual Value Unknown - $\mu\text{g}/\mu\text{l}$</u>	<u>Residual Error - $\mu\text{g}/\mu\text{l}$</u>
1	7	0.25	0.21	0.04
2	8	0.50	0.58	0.08
3	6	0.20	0.21	0.01
4	4	1.50	1.56	0.06
5	5	0.38	0.35	0.03
6	3	1.00	1.00	0.00
7	3	0.75	0.75	0.00
8	3	0.50	0.52	0.02
9	5	0.38	0.41	0.03
10	5	1.00	0.95	0.05
11	5	0.20	0.21	0.01
12	5	0.75	0.79	0.04
13	3	0.38	0.36	0.02
14	5	0.25	0.26	0.01
15	4	0.38	0.37	0.01
16	5	0.20	0.21	0.01
17	4	0.75	0.74	0.01
18	3	0.38	0.44	0.06
19	4	0.20	0.21	0.01
20	5	0.50	0.47	0.03
21	3	0.75	0.75	0.00
22	3	1.00	1.01	0.01
23	4	0.38	0.39	0.01

Table 2. (Cont'd)

24	3	0.50	0.51	0.01
25	4	0.20	0.19	0.01

Table 3. Accuracy Determinations for Dermatan Sulfate.

<u>Experiment Number</u>	<u>Number of Points Per Line</u>	<u>Predicted Value Unknown - $\mu\text{g}/\mu\text{l}$</u>	<u>Actual Value Unknown - $\mu\text{g}/\mu\text{l}$</u>	<u>Residual Error - $\mu\text{g}/\mu\text{l}$</u>
1	6	0.38	0.45	0.07
2	6	1.00	0.98	0.02
3	6	0.25	0.23	0.02
4	5	0.50	0.49	0.01
5	6	0.75	0.77	0.02
6	5	0.25	0.21	0.04
7	4	0.20	0.17	0.03
8	4	0.38	0.41	0.03
9	4	0.50	0.54	0.04
10	3	0.75	0.75	0.00
11	4	0.20	0.20	0.00
12	6	1.00	0.95	0.05
13	4	0.38	0.36	0.02
14	4	0.50	0.52	0.02
15	4	0.50	0.49	0.01
16	4	0.38	0.40	0.02
17	4	0.25	0.25	0.00
18	3	.075	0.74	0.01
19	4	0.50	0.47	0.03
20	4	0.20	0.20	0.00
21	3	0.25	0.25	0.00
22	4	0.38	0.37	0.01
23	3	1.00	0.99	0.01

Table 3. (Cont'd)

24	3	0.75	0.74	0.01
25	4	0.50	0.49	0.01

Table 4. Accuracy Determinations for Chondroitin 4-Sulfate.

<u>Experiment Number</u>	<u>Number of Points/Line</u>	<u>Predicted Value $\mu\text{g}/\mu\text{l}$</u>	<u>Actual Value $\mu\text{g}/\mu\text{l}$</u>	<u>Residual Error $\mu\text{g}/\mu\text{l}$</u>
1	5	10.00	10.11	0.11
2	4	5.00	5.43	0.43
3	5	10.00	9.86	0.14
4	5	15.00	14.32	0.68
5	5	2.50	2.27	0.23
6	4	7.50	7.99	0.49
7	4	20.00	20.06	0.06
8	3	5.00	5.14	0.14
9	3	10.00	9.81	0.19
10	4	2.50	1.80	0.70
11	3	15.00	15.15	0.15
12	4	7.50	7.79	0.29
13	4	5.00	4.51	0.49
14	5	20.00	19.66	0.34
15	4	15.00	15.13	0.13
16	4	2.50	2.41	0.09
17	3	7.50	7.74	0.24
18	4	10.00	10.68	0.68
19	5	5.00	5.05	0.05
20	4	20.00	20.23	0.23
21	3	2.50	2.58	0.08
22	3	15.00	15.04	0.04
23	5	7.50	6.97	0.53

Table 4. (cont'd)

24	4	10.00	10.71	0.71
25	5	5.00	5.82	0.82

Table 5. Accuracy Determinations for Chondroitin 6-Sulfate.

<u>Experiment Number</u>	<u>Number of Points/Line</u>	<u>Predicted Value $\mu\text{g}/\mu\text{l}$</u>	<u>Actual Value $\mu\text{g}/\mu\text{l}$</u>	<u>Residual Error $\mu\text{g}/\mu\text{l}$</u>
1	5	2.50	2.75	0.25
2	5	7.50	7.12	0.38
3	5	20.00	20.32	0.32
4	3	15.00	15.01	0.01
5	4	5.00	4.81	0.19
6	3	10.00	10.11	0.11
7	4	2.50	2.81	0.31
8	3	7.50	7.34	0.16
9	4	20.00	19.33	0.67
10	4	15.00	15.74	0.74
11	3	5.00	5.04	0.04
12	5	2.50	2.57	0.07
13	6	10.00	9.68	0.32
14	5	7.50	6.91	0.59
15	5	20.00	20.13	0.13
16	4	15.00	15.17	0.17
17	4	2.50	2.26	0.24
18	3	7.50	7.27	0.23
19	3	10.00	8.90	1.10
20	3	20.00	19.95	0.05
21	3	15.00	15.57	0.57
22	3	7.50	7.22	0.28
23	5	15.00	15.14	0.14

Table 5. (Cont'd)

24	3	5.00	4.87	0.13
25	5	2.50	2.61	0.11

Table 6. Precision Determinations for Hyaluronic Acid.

<u>Experiment Number</u>	<u>Number of Replicates</u>	<u>Mean Peak Area</u>	<u>Mean Deviation</u>
1	5	87.4	6.2
2	5	77.8	0.7
3	4	25.3	2.3
4	5	77.2	4.7
5	4	49.5	3.3
6	5	31.6	1.7
7	4	106.8	2.8
8	5	58.2	3.0
9	4	72.8	0.6
10	4	104.0	4.8
11	5	61.2	1.4
12	5	83.4	2.7
13	4	82.5	2.3
14	4	47.5	3.5
15	5	119.6	3.5
16	5	87.8	3.0
17	4	55.0	1.2
18	4	47.1	2.1
19	5	101.1	6.7
20	5	34.1	1.4
21	5	43.6	1.7
22	6	119.6	2.6

Table 6. (Cont'd).

23	6	55.8	2.3
24	4	66.8	0.8
25	4	106.7	0.8

Table 7. Precision Determination for Heparitin Sulfate.

<u>Experiment Number</u>	<u>Number of Replicates</u>	<u>Mean Peak Area</u>	<u>Mean Deviation</u>
1	5	128.8	9.4
2	6	227.7	19.4
3	6	263.3	21.6
4	4	171.2	1.4
5	6	133.0	19.7
6	5	146.7	8.4
7	5	182.3	25.4
8	4	251.0	11.0
9	6	234.6	9.9
10	4	207.3	8.1
11	5	237.9	6.1
12	5	242.6	7.1
13	4	212.0	8.0
14	6	171.8	14.9
15	6	134.1	7.4
16	5	252.3	8.4
17	6	146.8	14.4
18	5	174.6	9.1
19	4	99.5	4.2
20	5	232.4	21.7
21	4	327.6	10.1
22	4	104.1	1.4

Table 7. (Cont'd).

23	6	169.8	4.2
24	4	165.9	2.4
25	6	145.9	4.3

Table 8. Precision Determinations for Dermatan Sulfate.

<u>Experiment Number</u>	<u>Number of Replicates</u>	<u>Mean Peak Area</u>	<u>Mean Deviation</u>
1	4	157.5	8.2
2	4	256.9	3.8
3	6	232.1	16.6
4	5	187.1	11.9
5	6	197.0	11.8
6	4	224.6	5.9
7	4	163.4	2.9
8	5	278.0	13.8
9	6	210.7	12.3
10	5	224.5	19.2
11	6	233.8	8.8
12	5	306.2	14.2
13	6	278.1	19.4
14	4	350.2	16.9
15	5	313.0	12.4
16	6	183.0	9.3
17	4	155.6	16.2
18	4	333.7	15.0
19	5	157.1	14.4
20	5	205.7	8.6
21	6	309.3	16.3
22	6	217.6	10.7

Table 8. (Cont'd).

23	5	203.3	9.2
24	6	235.8	8.4
25	6	222.9	11.7

Table 9. Precision Determinations for Chondroitin 4-Sulfate.

<u>Experiment Number</u>	<u>Number of Replicates</u>	<u>Mean Optical Density</u> ¹	<u>Mean Deviation</u>
1	3	0.461	0.014
2	3	0.560	0.020
3	3	0.441	0.012
4	3	0.469	0.012
5	3	0.743	0.004
6	3	0.457	0.016
7	3	0.437	0.011
8	3	0.693	0.033
9	3	0.400	0.000
10	3	0.372	0.027
11	4	0.165	0.020
12	3	0.348	0.016
13	4	0.153	0.024
14	3	0.232	0.009
15	4	0.680	0.013
16	4	0.313	0.017
17	3	0.633	0.012
18	4	0.284	0.005
19	3	0.933	0.018
20	4	0.845	0.020
21	3	0.439	0.017
22	4	0.393	0.005

¹
Absorbance.

Table 9. (Cont'd).

23	4	0.612	0.014
24	3	0.957	0.004
25	3	0.640	0.030

Table 10. Precision Determinations for Chondroitin 6-Sulfate.

<u>Experiment Number</u>	<u>Number of Replicates</u>	<u>Mean Optical Density</u> ¹	<u>Mean Deviation</u>
1	3	0.495	0.005
2	3	0.427	0.007
3	3	0.294	0.002
4	3	0.317	0.010
5	3	0.609	0.047
6	3	0.395	0.008
7	3	0.340	0.003
8	3	0.240	0.012
9	3	0.423	0.015
10	3	0.154	0.020
11	3	0.629	0.007
12	4	0.301	0.016
13	4	0.083	0.009
14	4	0.268	0.017
15	4	0.110	0.005
16	4	0.207	0.008
17	3	0.544	0.012
18	3	0.246	0.001
19	4	0.208	0.004
20	3	0.502	0.006
21	3	0.586	0.013
22	3	0.578	0.006

¹
Absorbance.

Table 10. (Cont'd).

23	3	0.721	0.012
24	3	0.541	0.014
25	4	0.396	0.011

Table 11. Determination of Total Glycosaminoglycan by Two Methods.

<u>Experiment Number</u>	<u>ug/ul by Orcinol Procedure</u>	<u>ug/ul by Elson- Morgan Procedure</u>	<u>Difference</u>
1	6.45	7.56	1.11
	6.95	7.11	0.16
	4.62	4.74	0.12
	4.70	4.19	0.51
2	6.27	7.29	1.02
	3.91	4.37	0.46
	5.61	5.58	0.54
	4.05	5.04	0.99
3	6.59	6.50	0.09
	5.63	5.33	0.30
4	7.83	8.22	0.39
	5.50	5.38	0.12
	5.05	4.75	0.30
	4.51	4.17	0.34
Mean	5.55	5.73	0.18

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