1983

Articular Cartilage Proteoglycans In Normal And Osteoarthritic Mice.

Katherine S. Rostand
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ARTICULAR CARTILAGE PROTEOGLYCANS IN NORMAL AND OSTEOARTHRITIC MICE

The University of Alabama in Birmingham PH.D. 1983

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ARTICULAR CARTILAGE PROTEOGLYCANS IN NORMAL AND OSTEOARTHRITIC MICE

by

KATHERINE S. ROSTAND

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in The Graduate School, University of Alabama in Birmingham

BIRMINGHAM, ALABAMA

1982

ABSTRACT OF DISSERTATION

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Title Articular Cartilage Proteoglycans in Normal and Osteoarthritic Mice

Osteoarthritis (OA) is a non-inflammatory, degenerative disease of joints, such as the knee and hip, in which loss of articular cartilage results in loss of joint function, pain, and may eventually necessitate joint replacement. Although specimens of human articular cartilage have been studied *in vitro*, animal models of the disease offer the best opportunity to look carefully at the earliest biochemical events in the development of OA. Because of the high, spontaneous incidence of OA in the knee joint of the highly inbred mouse strain, STR/1N, it has been used in this study to examine articular cartilage proteoglycans (PG) in the disease. To characterize the PG of mouse articular cartilage, standard methods have been modified to facilitate processing the small amounts of cartilage available from knee joints of mice. *In vivo* labelling with ^{35}S-sulfate has permitted detection of newly synthesized mouse PG and several precautions have been taken to minimize degradation during extraction and isolation of the cartilage PG.

The ^{35}S-PG from normal and OA mice were compared with respect to: (1) extractability from the cartilage by 0.4 M and 4.0 M GuHCl; (2) ability to aggregate and size of monomer as judged by chromatography on Sepharose CL-2B; (3) size of glycosaminoglycan (GAG) chains; (4) level of sulfation of GAG as determined by CPG-DEAE chromatography; (5) sensitivity of GAG to chondroitinase AC and ABC; (6) relative amounts of chondroitin-4-sulfate and chondroitin-6-sulfate after HPLC separation of the isomeric sulfated disaccharides produced by chondroitinase digestion; and (7) immunologic identification of the link protein(s). Although there was a significant increase in the total amount of extractable ^{35}S-PG from the OA cartilage, no PG abnormalities were detected by these analyses. Nevertheless, the STR/1N mouse strain remains an interesting model for OA. Evaluation of the hormonal status of these animals particularly their levels of growth hormone, may provide clues to the etiology of OA.

Abstract Approved by: Committee Chairman Tony R. Baker

Program Director R. D. Wells

Date 3/18/83 Dean of Graduate School Robert Koozer

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I am especially grateful to Caroline Dunn for her advice and expertise in the final preparation of this dissertation.

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LIST OF ABBREVIATIONS

BNC	bovine nasal cartilage
BAC	bovine articular cartilage
C-4-S	chondroitin-4-sulfate
C-6-S	chondroitin-6-sulfate
CPG	controlled-pore glass
CS	chondroitin sulfate
DEAE	diethylaminoethyl
ΔDi-0S	2-acetamido-2-deoxy-3-0-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose
ΔDi-4S	2-acetamido-2-deoxy-3-0-(β-D-gluco-4-enepyranosyluronic acid)-4-0-sulfo-D-galactose
ΔDi-6S	2-acetamido-2-deoxy-3-0-(β-D-gluco-4-enepyranosyluronic acid)-6-0-sulfo-D-galactose
EDTA	ethylenediaminetetraacetate
fBAC	fetal bovine articular cartilage
GAG	glycosaminoglycan(s)
GuCl	guanidine hydrochloride
HA	hyaluronic acid
KS	keratan sulfate
LP	link protein(s)
OA	osteoarthritis
PG	proteoglycan(s)
SAC	sheep articular cartilage

LIST OF ABBREVIATIONS (Continued)

SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
PAPS	3'-phosphoadenosine 5'-phosphosulfate
UA	uronic acid

INTRODUCTION

Articular Cartilage

Cartilage is one of the essential weight-bearing components of the human skeleton and forms the articulating surface of joints. This articular cartilage is classified as hyaline cartilage since it lacks elastic fibers and has less collagen than the fibrocartilages. The major structures seen in an electron micrograph of a thin section of articular cartilage are an abundant intercellular matrix, composed of fibers and an interspersed 'ground substance,' and a single cell-type, the chondrocyte (Figure 1); there are no nerves nor is the cartilage vascularized. Unlike other hyaline cartilages, articular cartilage is not covered by perichondrium which is a source of new chondrocytes and most observers agree that mitoses cannot be found in normal adult articular cartilage (Stockwell & Meachim, 1979). Thus, at the end of skeletal growth, the chondrocytes in the articular cartilage ($\sim 15 \times 10^3$ cells per mm^3 in human femoral condyle, Stockwell, 1971) are responsible for the maintenance of the matrix throughout the rest of the individual's life.

The suitability of articular cartilage as a load-bearing, moving surface of joints depends primarily on the physical and biochemical integrity of the extracellular matrix (Meachim & Stockwell, 1979;

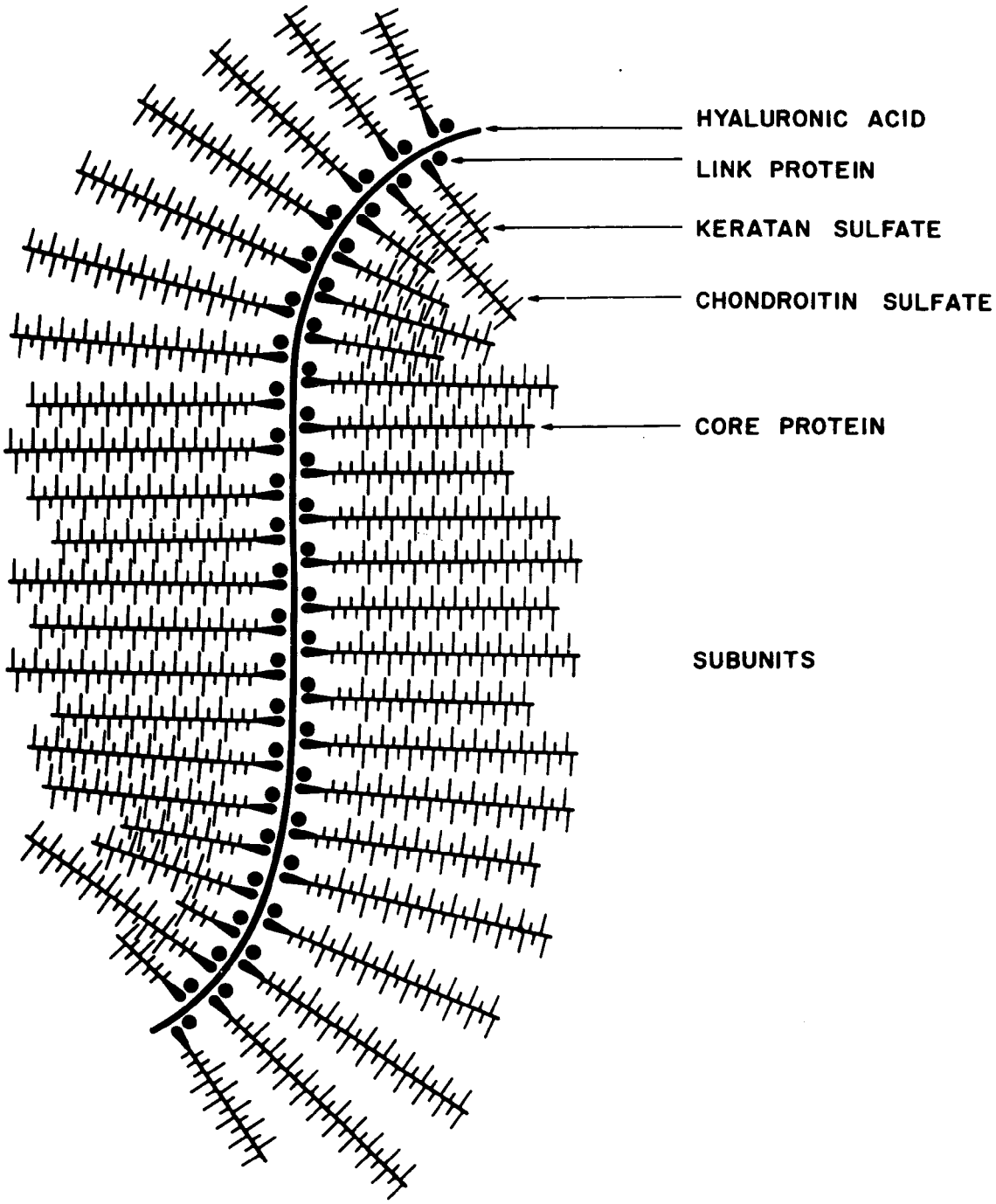
Figure 1 Electron micrograph of chondrocytes in their extracellular matrix. Thin section from mouse articular cartilage, tibial surface. Tissue was fixed with 2% OsO_4 and stained by the high iron diamine-thiocarbohydrazide-silver proteinate method, HID-TCH-SP (Takagi, Parmley, & Denys, 1981). HID stains sulfated glycosaminoglycans; TCH-SP enhances HID staining. N = nucleus of chondrocytes, arrow indicates a vesicle in cell containing electron-dense granules. Magnification x12,500.

Bayliss & Venn, 1980). In adult articular cartilage 75-78% of the wet weight is water; about one-half the dry weight is Type II collagen, and one-half is cartilage specific proteoglycans (PG) plus other non-collagenous proteins. The collagen fibers provide a network that defines tissue shape and resists tensile forces, while the proteoglycan provides an hydrated, viscous gel that absorbs compressive loads.

Structure of Cartilage Proteoglycans

Most cartilage proteoglycans exist in the matrix as large aggregates consisting of three components, the PG monomers, hyaluronic acid (HA), and 1 or 2 link proteins (Figure 2). The average size of the aggregates is primarily dependent upon the length of the HA filament and can vary for different cartilages. Aggregates of approximately 50 million molecular weight exist in bovine nasal cartilage (Hascall & Heinegard, 1974; Rosenberg, Hellman, & Kleinschmidt, 1975), but those from articular cartilage are smaller, as is the size of the monomer when compared to monomer from bovine nasal cartilage. The two link proteins, 1 and 2, have molecular weights of 46,000 and 40,500, respectively (Baker & Caterson, 1979). Link 1 has more covalently bound carbohydrate than link 2 (9.5% vs. 3.0%), but their amino acid compositions are similar and probably their sequences are similar (Baker & Caterson, 1979). Both link proteins interact with HA alone (Oegema, Brown, & Dziewiatkowski, 1977) and with the HA-binding region of the PG monomer (Caterson & Baker, 1978; Heinegard & Hascall, 1979). Although PG monomers and HA can specifically associate to form a

Figure 2 The aggregate structure of cartilage proteoglycans. (Rosenberg, Hellman, & Kleinschmidt, 1975)


HYALURONIC ACID
LINK PROTEIN
KERATAN SULFATE
CHONDROITIN SULFATE
CORE PROTEIN
SUBUNITS

complex, the interaction of the link proteins with the complex stabilizes the aggregate making it more resistant to dissociation by low pH and elevated temperature (Keiser, Shulman, & Sanderson, 1972; Hardingham, 1979).

Most of what is known about the structure of the PG monomer has come from extensive studies on PG from bovine nasal septum and a transplantable rat chondrosarcoma (Hascall & Hascall, 1981). These macromolecules consist of a core protein (MW ≅ 250,000) to which a large number of chondroitin sulfate (CS) and keratan sulfate (KS) chains are covalently attached (Figure 3). An average of about 100 CS chains (MW ≅ 20,000) are present. Each CS chain is a linear polymer of the repeating disaccharide,

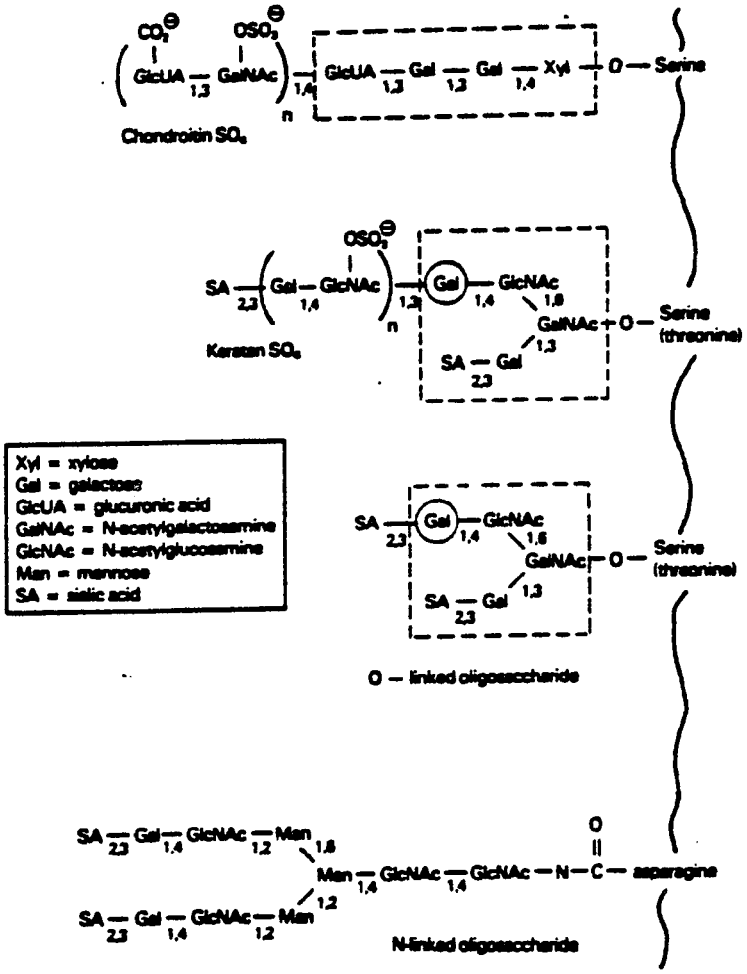
[glucuronate-β 1,3-N-acetylgalactosamine-β 1,4-]
|
4 or 6 sulfate

the GalNAc may be sulfated in either the 4- or 6-position. The chains are attached to the core protein through a glycosidic bond between the hydroxyl group of a serine and a xylose residue at the reducing end of the chain (Rodén & Smith, 1966). Keratan sulfate chains (MW ≅ 5,000) are linear polymers of the repeating disaccharide,

[N-acetylglucosamine-β 1,3-galactose-β 1,4]-
|
6-sulfate

and are bound to the core protein through an oligosaccharide structure which in turn is linked to the protein by a glycosidic bond between N-acetylgalactosamine and the hydroxyl of serine or threonine. Not all of these O-linked oligosaccharides have KS chains covalently

Figure 3 Chemical structure of proteoglycan monomer from cartilage. (Hascall & Hascall, 1981)


Chondroitin SO4
Keratan SO4
Xyl = xylose
Gal = galactose
GlcUA = glucuronic acid
GalNAc = N-acetylgalactosamine
GlcNAc = N-acetylglucosamine
Man = mannose
SA = sialic acid
O – linked oligosaccharide
N-linked oligosaccharide
Serine (threonine)
asparagine

attached, but the total number of KS chains plus O-linked oligosaccharides remains relatively constant, about 100 per core protein. The relative proportion of O-linked oligosaccharides to KS chains varies with age of the cartilage; mature cartilages having more KS chains than O-linked oligosaccharides, embryonic cartilages having a higher proportion of O-linked oligosaccharides relative to KS chains. Most of the KS chains plus O-linked oligosaccharides are bound in the middle region of the core protein (KS-attachment region) while most of the CS chains are concentrated at one end (CS-attachment region). The other end of the core protein contains few or no attached GAG chains and contains the active site that binds to HA (HA-binding region) through a noncovalent, highly specific interaction. The HA-binding region of the core protein contains oligosaccharides covalently bound by N-glycosylamine linkages between N-acetylglucosamine and asparagine. There are about 15 of these N-linked oligosaccharides per core protein and they contain primarily sialic acid, galactose, mannose, and N-acetylglucosamine with a small amount of fucose.

It is not difficult to imagine that the complexity of the PG monomer structure could give rise to great polydispersity and heterogeneity in any given preparation of PG. Indeed, PG preparations do appear to contain a variety of structures with differences in molecular size, chemical composition (relative CS, KS, and protein content) and ability to aggregate. Eighty-five percent of the total extractable PG are aggregating proteoglycans, but are very polydisperse in size. Hascall and Sajdera (1970) suggested that the source of this polydispersity is a variable number of CS chains attached to the PG

protein core. However, Hopwood and Robinson (1975), analyzing a similar PG fraction, found a constant number of CS chains per PG molecule, but a different average chain length. Furthermore, they proposed that the average CS chain length was determined by the relative proportion of three different CS species. Finally, sub-fractions with different amino acid compositions of the protein core were identified. Although not confirmed by others, these results suggested that there exist, at least in nasal cartilage, three distinct PG pools.

Another population of PG, the non-aggregating proteoglycans, representing about 10-15% of the total extractable PG, cannot interact with HA (Heinegard & Hascall, 1979). Although these molecules do not interact with HA, they contain proportionally more protein and less CS than the aggregating PG monomers (Heinegard & Hascall, 1979). Oegema (1980) has implied that these PG may be precursors of aggregating PG.

A third population of PG from cartilage has been described by Heinegard, Paulsson, Inerot, and Carlström (1981). These PG monomers contain a protein core that is monodisperse, in contrast with the extreme polydispersity of other PG. These low-molecular-weight PG (MW = 76,300) constitute less than 2% (by weight) of the total extracted PG, are not capable of interacting with HA, and contain only 2-3 large CS chains.

The function(s) of the non-aggregating and the low-molecular weight PG is not known. They do not appear to be specific to BNC cartilage (Heinegard et al., 1981; Heinegard & Hascall, 1979).

Lastly, two types of high molecular weight, aggregating PG have been recognized (Heinegard, Paulsson, & Sommarin, 1982). One type is chondroitin sulfate rich and predominates in young cartilage, while the other is keratan sulfate rich and predominates in older cartilage. Precisely how their structures differ from each other and whether their functions differ is not known.

Function of Proteoglycans in Articular Cartilage

Proteoglycans occupy large molecular domains in solution due primarily to the extended lengths of the CS chains. The molecules are reversibly compressible. When a compressive load is applied, and solvent is displaced from the molecular domain, the interactions between the bound, like-charged GAG chains increase. When the load is removed, the molecules expand to minimize the intramolecular interactions and maximize solvation. This property of proteoglycans is critical for normal function of articular cartilage which must act as a cushion for changing, compressive loads. In addition to the intramolecular interactions, there is intermolecular competition between PG molecules for available solvent in the matrix. (In normal articular cartilage, the concentration of PG is three to five times more than would be possible if the molecules were fully extended and solvated.) This competition serves to reduce the domains of the molecules, thus increasing the charge density. These partially compressed molecules have been compared to a partially compressed spring (Hascall & Hascall, 1981) in that they resist a compressive load with less total

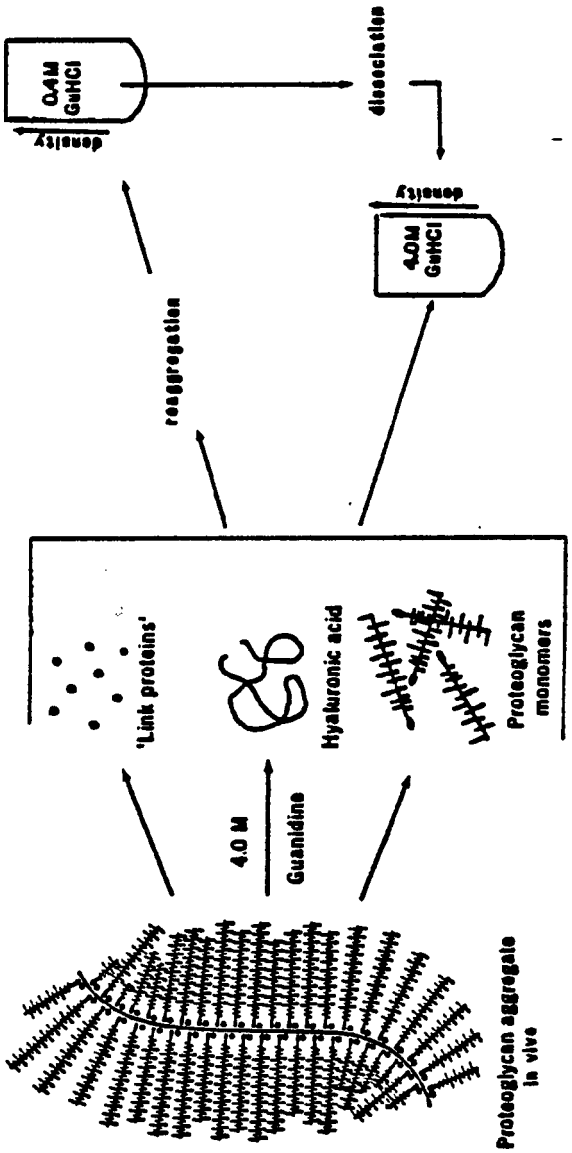
deformation than a fully extended molecule. Thus, not only the structure of the PG, but its concentration in the matrix as well, affects the function of articular cartilage.

Isolation of Proteoglycans

Although Franek and Dunstone (1966) were the first to use isopycnic density gradient centrifugation methods to purify proteoglycans, Hascall and Sajdera (1969) first developed the basic two-step procedure for the extraction and isolation of cartilage proteoglycans (Figure 4). In general, these two steps are: (1) extraction with a chaotropic agent which dissociates the aggregate into its components and from its entrapment (association) with the collagen network, (2) fractionation by CsCl equilibrium density gradient centrifugation. The procedure as it has been generally applied to cartilage proteoglycans is described below; modifications for applications to other tissues are reviewed by Hascall and Kimura (1982).

The dissociating agent generally used for extraction is 4 M guanidine hydrochloride (GuHCl). The extractant solution is buffered by sodium acetate at pH 6.0-6.5, above the pH for optimum activity of acid proteases and below the optimum for neutral proteases. Other means of minimizing degradation of the proteoglycan during extraction include keeping the tissue and solutions at 4°C and the inclusion of several protease inhibitors in the extraction mixture. The inhibitors frequently used are 6-aminohexanoic acid (for cathepsin D activity), benzamidine-HCl (for trypsin-like activity), EDTA (for metalloproteases), phenylmethylsulfonylfluoride (for serine-dependent protease),

Figure 4 Extraction and isolation of cartilage proteoglycans.


0.4M GuHCl
density
dissociation
density
4.0M GuHCl
reaggregation
'Link proteins'
Hyaluronic acid
Proteoglycan monomers
4.0 M
Guanidine
Proteoglycan aggregate in vivo

and iodoacetamide (for thiol-dependent proteases). Thin cartilage slices or a milled powder is extracted with 5-15 volumes of the 4 M GuHCl-inhibitor 'cocktail'; the mixture is stirred, at 4°C, for as long as 24 h. The extract is clarified by filtering or by a brief centrifugation, CsCl is added to a density of 1.65 g/ml, and an equilibrium density gradient established in the ultracentrifuge; this requires approximately 48 h at ≧ 100,000 x g. Substances in the extract that have low buoyant densities, including the link proteins of PG aggregate, float to the top of the gradient. The PG monomer, which has a high ratio of GAG to protein and therefore a high buoyant density, is recovered at the bottom of the gradient (D1). Eighty to 90 percent of the PG from bovine nasal cartilage can be recovered in this way. If the 4 M GuHCl extract is first dialyzed to associative conditions (0.4 M GuHCl), PG aggregate (monomer + HA + link proteins) accumulates in the bottom (A1) of the gradient. This A1 fraction can again be dissociated in 4 M GuHCl and the aggregate components fractionated in a dissociative CsCl equilibrium density gradient. The PG monomer is again recovered at the bottom (A1D1) of the gradient; HA is found primarily in the middle densities, and the least dense link proteins float to the top. This top fraction has been used as the starting material for further purification of the link proteins and the separation of link 1 and link 2 (Baker & Caterson, 1979). The A1D1 fraction may contain, in addition to intact PG monomer, any highly sulfated degradation products from the PG, such as free CS chains or CS-attachment region. The appropriate fractions from the CsCl density

gradient are collected, dialyzed exhaustively against water, lyophilized, and used for further characterization of the aggregate and its components.

Biosynthesis of Cartilage Proteoglycans

The chondrocyte is responsible for the synthesis and degradation of the matrix components of articular cartilage. Although it is a highly differentiated cell, the chondrocyte possesses the usual complement of cell structures and organelles, including plasma membrane, nucleus and nucleolus, rough endoplasmic reticulum, Golgi apparatus, mitochondria, microtubules, and lysosomes. The Golgi is the site of addition of most of the glycosaminoglycan and oligosaccharide chains to the core protein. The chondrocyte's metabolism is chiefly anaerobic; the oxygen uptake per cell has been measured to be ~ 1/50 that of a liver or kidney cell. Although lactic acid is released as a metabolic end product, the pH of the matrix is between 6.95 - 7.4.

Unlike many other mammalian cells the chondrocyte in articular cartilage does not experience much cell-cell interaction and the matrix itself influences the biosynthetic activity of the cells. Chondrocytes in culture incorporate ^{35}S-sulfate into macromolecular sulfated proteoglycans which are released into the medium as soluble aggregates or in some cases deposited as nodules of extracellular matrix. The proteoglycans are the predominant, if not the only, sulfated molecules the cells are synthesizing and the sulfate incorporation is therefore a reliable index of PG synthesis. This incorporation is specifically inhibited by HA in the culture medium (Wiebkin &

Muir, 1973), but not by complexes of HA and PG monomer (Wiebkin, Hardingham, & Muir, 1975). These data and other binding studies (Wiebkin & Muir, 1975), suggest that there are cell surface receptors on chondrocytes that specifically bind extracellular HA and this binding in some way influences the synthesis of proteoglycans. It is interesting to speculate that these cell surface receptors may contain specificities similar to the HA-binding region of the core protein (Wiebkin et al., 1975).

Chondrocytes have been thought to be rather inert, isolated cells. In adult cartilage proteoglycans turn-over slowly and the collagen appears to turn-over even less. But the cells are capable of responding to external factors and of altering the steady-state balance of synthesis and degradation. Studies have shown they respond to insulin (Salmon, Duvall, & Thompson, 1968; McCumbee & Lebovitz, 1980), somatomedins (Phillips & Vassilopoulos-Sellin, 1980) and even growth hormone directly (Isaksson, Jansson, & Gause, 1982). The response to other so-called anabolic and catabolic hormones can be demonstrated by ultrastructural changes in the cell that are consistent with the known physiologic effect of the hormone (Silberberg, 1968). The responsiveness of the chondrocyte is an important factor in maintaining normal, functional articular cartilage. The cells must be able to maintain high concentrations of proteoglycans in the matrix and to reproduce faithfully the structural features of the PG (e.g., normal complement of GAG) necessary to proper function. Whether or not the articular cartilage chondrocytes do this in the pathological condition of degenerative joint disease has been the subject of many studies (Table 1).

TABLE 1

BIOCHEMICAL CHANGES IN CARTILAGE PROTEOGLYCANS IN DEGENERATIVE JOINT DISEASE

Analyses	Biochemical Changes Compared to Normal[1]	References
Metabolic Activity of Chondrocytes	Increased ^{3}H-thymidine incorporation in vitro	Telhag (1976), Hans (1976), Mitchell & Shepard (1981)
	Increased ^{35}S-sulfate incorporation in vitro	Bollett & Nance (1966), Mankin & Lipiello (1970), Mankin et al., (1971)
Cartilage Composition	Increased CS content in early osteoarthritis	Benmaman et al. (1969), Mankin & Lippiello (1971), McDevitt & Muir (1974, 1976), Sweet et al. (1977)
	Total glycosaminoglycan decreased in osteoarthritis	Bollet & Nance (1966), Inerot et al. (1978)
	Total glycosaminoglycan reduced in fibrillated areas	Sweet et al. (1977)
	Increased glycosaminoglycan content in osteophytic areas	Sweet et al. (1977)
	Increased water content	Bollet & Nance (1966), Mankin & Thrasher (1975), McDevitt & Muir (1976), Maroudas (1976), Inerot et al. (1978)
Proteoglycan Content	Increased in early osteoarthritis	McDevitt & Muir (1976)
CS/KS[2] Ratio	Increased in early stages of the disease	McDevitt & Muir (1974, 1976), McDevitt et al. (1977), Sweet et al., (1977), Michelacci et al. (1979)
C-4-S/C-6-S Ratio	More C-4-S proteoglycan synthesized in osteoarthritis	Mankin & Lippiello (1971), Michelacci et al. (1979)
CS Chain Size	No change in size distribution	Inerot et al. (1978)
	Decreased CS length	Bollet & Nance (1966)
Extractability	More proteoglycans extracted in associative extracts	McDevitt & Muir (1976), Brandt (1974, 1977), Sweet et al. (1977), Moskowitz et al. (1979)
	More proteoglycan extracted in dissociative extracts	McDevitt & Muit (1974), Sweet et al. (1977), Muit (1977), Muit (1977), Inerot et al. (1978)
	Relatively more KS remains in the cartilage residue after extraction	Inerot et al. (1978)
Proteoglycan Aggregates	Absent in osteoarthritis	Palmoski & Brandt (1976), Perricone et al. (1977), Moskowitz et al. (1979)
	Present but proportionally less compared to proteoglycan monomer	Brandt & Palmoski (1976), McDevitt et al. (1977), Muir (1977), Inerot et al. (1978)
Proteoglycan Monomers	Smaller in size	McDevitt & Muir (1979)
	Unchanged in size	Moskowitz et al. (1979), McDevitt & Muir (1979)
	Smaller	Sweet et al. (1977), Inerot et al. (1978)
	Contain less total carbohydrate	Sweet et al. (1977)
	Possess functional HA-binding regions	Brandt et al. (1976), Brandt (1977)
	Lack functional HA-binding regions	McDevitt & Muir (1976), Palmoski & Brandt (1976), Perricone et al. (1977), Brandt (1977), Inerot et al. (1978)
Hyaluronic Acid	Reduced amounts of HA in osteoarthritic cartilage	Sweet et al. (1977)
Link Proteins	Absent in proteoglycan extracts	Perricone et al. (1977)

[1] The term "normal" cartilage means in some cases that samples came from joints without detectable osteoarthritis. In others, the samples came from "apparently normal" areas on the same joint, and in some experiments "normals" came from either sham operated or control joints of experimental animal models for osteorathritis.

[2] Abbreviations - CS, chondroitin sulfate; C-4-S, chondroitin-4-sulfate; C-6-S, chondroitin-6-sulfate; KS, Keratan sulfate; HA, hyaluronic acid.

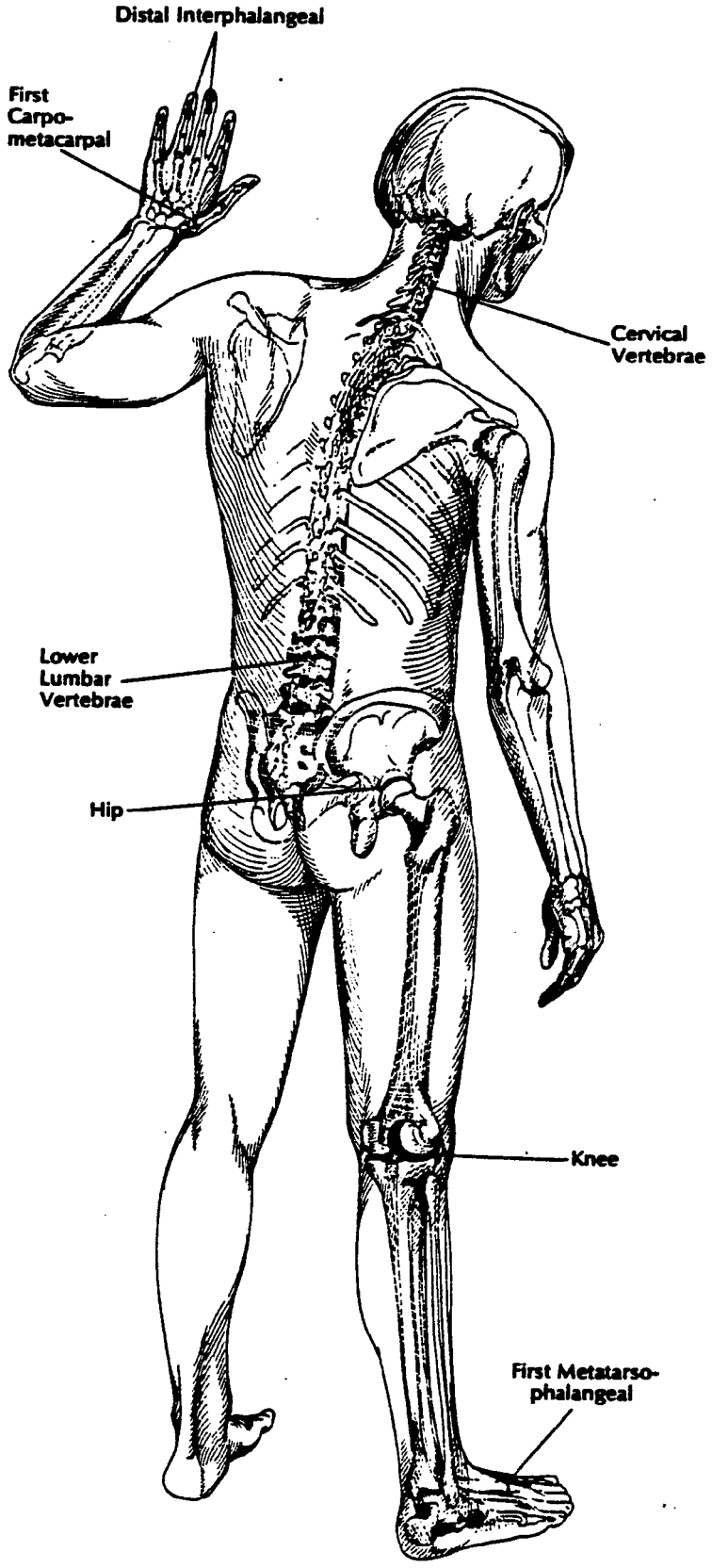
Degenerative Joint Disease/Osteoarthritis

Clinical Manifestations in Humans

Osteoarthritis is often considered to be an aging phenomenon since it usually occurs in older individuals. Symptoms are uncommon before 40 years of age, although radiological signs may be present as early as 20 years. In most instances the onset is insidious, with no systemic manifestations, the patient's chief complaint being joint pain during movement. In the early stages of the disease, "limbering-up" exercises may relieve joint stiffness, but, later, limitation of motion may become severe with pain even at rest. Certain joints are most often involved; these include the distal interphalangeal joints of the fingers, first carpometacarpal joint, the lower lumbar and cervical vertebrae, hip, knee, and first metatarsophalangeal (Figure 5). Radiological evaluation reveals the major clinical finding of osteoarthritis, namely, narrowing of the joint space due to loss of articular cartilage. Secondarily, osteophytes may develop and be visible at the joint margins. Inflammation of the joint tissues may be evident in the later stages and is probably a result of changes in the mechanics of joint movement. The clinical test for rheumatoid factor as well as other blood findings are normal (Moskowitz, 1972a, p. 1043).

By the time the disease is clinically evident, much cartilage has been lost from the articulating surfaces. There is no means of detecting the disease in its earliest stages and no known treatment can slow or reverse the degeneration of the cartilage. Treatment generally

Figure 5 Sites of osteoarthritic involvement in humans. (Moskowitz, R.W., 1979)


Distal Interphalangeal
First Carpo-metacarpal
Cervical Vertebrae
Lower Lumbar Vertebrae
Hip
Knee
First Metatarso-phalangeal

involves resting the affected joints and the administration of analgesics for pain, but surgical intervention to replace the joint may be required (Moskowitz, 1972b, p. 1065).

Biochemical Changes in the Cartilage Proteoglycans

Since the chemical and physical structure of proteoglycans is so important to the proper functioning of articular cartilage, these macromolecules have been the focus of many studies attempting to determine the biochemical events leading to cartilage degeneration in osteoarthritis. Most of the structural alterations that can be thought of a priori have been reported in one or another study (Table 1). In general, these studies have followed two approaches: 1. analysis of human articular cartilage taken from osteoarthritic joints at the time of joint replacements; 2. use of animal models in which the development of the cartilage degeneration appears to follow the pattern of the disease in humans.

Studies Using Human Articular Cartilage

The biochemistry of normal and osteoarthritic human articular cartilage has been reviewed recently by Bayliss and Venn (1980). One of the first truly biochemical studies of osteoarthritic articular cartilage was by Bollet and Nance in 1966. They attempted to differentiate between the loss of proteoglycan due to protease degradation (loss of both chondroitin sulfate and keratan sulfate) and loss due to hyaluronidase-type degradation of the chondroitin sulfate chains.

They reported a decreased CS chain-length in OA cartilage and a proportionally greater loss of CS over KS, suggesting a role of a "depolymerase such as hyaluronidase in chondroitin sulfate degradation in these lesions" (p. 1176). Their findings did not, however, rule-out a preferential synthesis of lower molecular weight CS. There was also a slight decrease in the concentration of neutral sugar (galactose) in OA cartilage so that proteolysis remained a possible mechanism of degradation. The water content of cartilage from the osteoarthritic lesions was higher than in normal articular cartilage, 75% (w/w) vs. 68% (w/w), respectively. The ash weights were not significantly different. In vitro incubations with ^{35}S-sulfate and ^{14}C-acetate resulted in an increased specific activity of the CS in osteoarthritic cartilage, suggesting increased turnover of the polysaccharide compared to normal cartilage. Bollet and Nance also compared normal specimens from individuals ranging in age from 30-80 years and found, in contrast to later studies, no correlation of age with concentration of CS, neutral sugar, water content, or ash content.

Following Bollet and Nance, investigators continued to compare the same parameters in studying normal and osteoarthritic cartilage but in addition were aware of the non-covalent interactions involved in proteoglycan aggregation and began to test for the ability of extracted proteoglycans to bind hyaluronic acid. In addition to determination of the presence of hyaluronic acid binding region, chromatography on columns of Sepharose CL-2B gel made possible the comparison of monomer sizes from normal and osteoarthritic cartilage.

Glycosaminoglycan chain sizes could also be readily compared by molecular sieve chromatography. Furthermore, the availability of purified chondroitinases, AC and ABC, allowed ready analysis for the presence or absence of dermatan sulfate and keratan sulfate as well as the relative amounts of chondroitin-4-sulfate and chondroitin-6-sulfate. Finally, the link proteins were becoming more fully characterized and could be studied.

The conflicting data in these studies of human articular cartilage probably result from two important aspects of experimental design. First, in 1975 Oegema emphasized the need to include protease inhibitors during extraction and isolation of proteoglycans. Studies before this time had not used protease inhibitors consistently and in fact some later studies did not take these precautions (Palmoski & Brandt, 1976; Perricone, Palmoski, & Brandt, 1977). Furthermore, extraction of proteoglycan from articular cartilage proved to be more difficult than from bovine nasal cartilage and frequently long extraction times were used thus increasing the risk of proteolysis. Subsequently, Bayliss and Venn (1980) have reported that extraction efficiency is optimized for articular cartilage if very thin tissue slices or milled cartilage is used.

A second variable in the studies on human articular cartilage is a result of topographical variation of the chemical composition of human femoral head cartilage (Venn, 1979). The water content, keratan sulfate content, chondroitin sulfate, and collagen content of the cartilage varies both with depth into the tissue and position on the articulating surface (cartilage thickness varies over the surface and

positional variation may reflect the depth variation). This makes it difficult to presuppose that a piece of normal-looking cartilage from one position on the articulating surface is a true control for the osteoarthritic lesion somewhere else on the surface. Furthermore, Venn suggests that it is not valid to assume, as some studies had, that the fibrillation seen at post-mortem is merely different in extent from those degenerative changes seen in osteoarthritis.

Despite the discrepancies, most studies did agree that the water content of osteoarthritic cartilage in early stages of the disease was greater than that of normal articular cartilage. Secondly, the in vitro incorporation studies suggested that chondrocytes maintain a considerable biosynthetic capacity despite their normally low activity in mature articular cartilage. Thus, the chondrocytes were capable of responding to a loss of proteoglycan from the matrix. However, the synthesis was not sufficient to replace and repair the cartilage. Furthermore, at least two studies (Mankin & Lippiello, 1971; Michelacci Mourao, Laredo, & Dietrich, 1979) suggested that the increase in C-4-S over C-6-S and decrease in KS content meant that the chondrocyte had reverted to a "chondroblastic" stage and was synthesizing an immature proteoglycan like that produced in earlier development. This proteoglycan was presumed less able to withstand the wear and tear of normal joint use, perhaps due to its decreased KS content (Kempson, Muir, Swanson, & Freeman, 1970).

In the end, the studies with human articular cartilage did little to clarify the situation. Studies with animal models seemed to offer

the best opportunity to look carefully at the very earliest biochemical events that could be detected before visible lesions appeared.

Studies Using Animal Models

Surgically induced models in the dog and the rabbit are probably the most often studied. In the dog, osteoarthritis has been induced by cutting the cruciate ligament. The ligament is cut in one knee; the contralateral knee is sham operated and used as the control. Considering that new stresses are put on this sham operated knee because of the instabilities in the experimental joint, it might be that it is not a valid control. In addition, it must be admitted that there is an element of inflammation that must be considered in any surgical procedure. Nevertheless, probably the most thorough study of osteoarthritis of the knee joint was by McDevitt and Muir using dogs with cut anterior cruciate ligaments. The changes that ensued after the surgery were found to be indistinguishable from those of natural OA in the dog and the model had two advantages: 1) the time of onset was known, 2) the lesion appeared in the same region of the tibial surface in every dog so that this area could be sampled before lesions developed and the subsequent events followed.

Three phases of this experimental osteoarthritis in the dog were recognized (Muir, 1977):

1. (1-3 weeks after surgery) Minimal histological changes are seen. Only a focal area of the medial condyle of the tibia is affected. Biochemical changes include increased water

content and extractability of the PG and increased molar ratio of galactosamine: glucosamine of the cartilage.

2. (3-12 weeks after surgery) The erosion of cartilage in focal area develops with some loss of H_2O and PG from this region. Phase 1 changes now occurring in remaining cartilage.
3. (12-48 weeks after surgery) Severe erosion of focal site occurs with concommitant decrease in water content and a marked loss of PG. Changes of Phase 2 are now seen throughout cartilage. Now recognizable by pathology or radiology as OA.

Contrary to other studies (Nimni & Deshmukh, 1973; Gay, Miller, Lemmen, Remberger, Matzen, & Kuhu, 1978), no evidence of a switch to Type I collagen synthesis by the chondrocytes was found.

Another informative study in dogs took advantage of the congenital hip dysplasia that is common in certain breeds of dogs (Inerot, Heinegard, Audell, and Olsson, 1978). Dogs of different ages were used and changes with age were described as well as a comparison of osteoarthritic cartilage with age-matched normals. The degenerated cartilage had a higher water content than normal cartilage of the same age. A higher proportion of the PG in osteoarthritic cartilage was extractable. The proteoglycan monomers from the OA samples were smaller with higher uronic acid/protein ratios and fewer were able to bind to hyaluronic acid to form aggregates. The average sizes of the chondroitin sulfate chains did not differ appreciably. Keratan sulfate content did not differ appreciably in degenerated cartilage as compared with normals of the same age, even though KS increased with

increasing age of the cartilage. This study emphasized the need for age-matched controls in studying changes in OA cartilage.

In rabbits after meniscectomy, the incidence of osteoarthritis has been variable and the progression of events not as clearly defined as in the dog studies. In addition, the extraction of the proteoglycan has been low (as little as 20%) and the reported loss of aggregation much more extreme than in other studies. A total loss of aggregation has been reported (Moskowitz, Howell, Goldberg, Muniz, & Pita, 1979). A recent study by Pita (1982) using highly purified collagenase on rabbit articular cartilage shows that 70% of the tissue hexuronate could be extracted by 0.5 M GuHCl after the collagenasetreatment and that 60% of this was present as aggregate. Without collagenase treatment only 20-30% was present as aggregate, although addition of exogenous hyaluronic acid resulted in conversion to 60% aggregates. Thus, it seems particularly difficult to extract all aggregate components efficiently from the rabbit articular cartilage and this could easily account for the diminished aggregation reported by others.

Other Animal Models

Surveys of animal species generally available for laboratory experimentation (Alspaugh and van Hoosier, 1973; Young, Fetter and Johnson, 1979) have revealed a varied incidence of osteoarthritis in the animal kingdom (Table 2).

The popular laboratory rat has a very limited incidence of the disease. OA occurs in the Syrian hamster but with a variable incidence. The guinea pig develops OA but only after 2½ years. The

TABLE 2

ANIMAL MODELS FOR OSTEOARTHRITIS

SPECIES	OCCURRENCE	REFERENCE
Dog	congenital hip dysplasia cut cruciate ligament	McDevitt & Muir, 1976; Inerot et al., 1978
Rabbit	cut cruciate ligament meniscectomy	Moskowitz, 1974; Telhag & Lindberg, 1972; Ehrlich, Mankin, Jones, Grossman, Crispen, & Ancona, 1975; Bohr, 1976
Hamster	Syrian breed; knees 31%-100%, 2nd yr.	Meachim & Illman, 1967; Silberberg, Saxon, & Sperling, 1952
Guinea pig	knees; 30-51 mo.	Silverstein & Sokoloff, 1958
Praomys (Mastomys) Natalensis	knees; 2nd yr.	Sokoloff, Snell, & Stewart, 1967
Rat	very limited	Sokoloff & Jay, 1956c
Mouse	STR/1N; knees > 90% males 12-17 mo.	Silberberg & Silberberg, 1950; Sokoloff, 1956

Mastomys, an African rodent which is larger than a rat and smaller than a rabbit, is a good candidate for an experimental model of OA but is not a familiar laboratory animal and is ill-tempered as well. Lastly, there are several inbred mouse strains which develop OA spontaneously and are attractive as animal models for the disease. The use of an inbred strain would help to minimize individual variation and facilitate the analysis of many animals at different ages.

The mouse strain STR/1N offers several advantages as a model for osteoarthritis. The incidence of OA in STR/1N is almost 100% for the males and is well-developed by 12 mo. The biochemical events would therefore occur during this period of time. In contrast, the C57 mouse strain develops osteoarthritis of the knee but with a variable incidence (Pataki, Rüttnu, & Abt, 1980). NZY/B1 and PN mice have rather low incidences of the disease, 68% at 18 months and 50% at 24 months, respectively (Wigley, Couchman, Maule, & Reay, 1977). Another advantage of STR/1N is the existence of the parent strain STR/N which has a much lower incidence of OA but is nevertheless closely related genetically (Table 3).

The STR/1N Mouse Strain

The STR/1N strain was isolated in 1951 by Dr. George E. Jay, Jr., as a spontaneous color mutant (Figure 6) of STR/N arising in the F_{29} generation. The STR/N mice were originally developed from the NHO strain of Strong by treatment with 3-methylcholanthrene.

TABLE 3

GENETIC PROFILE OF STR/N AND STR/1N MOUSE STRAINS

	3 * carbonic anhydrase	17 catalase	8 esterase	11 esterase	14 esterase	4 G-6-P dehydrogenase	7 glucose-P isomerase	5 glucuronidase	11 hemoglobin alpha-chain	7 hemoglobin beta-chain	1 isocitrate dehydrogenase	9 malic enzyme
	Car-2	Ce-2	Es-1	Es-3	Es-10	Gpd-1	Gpi-1	Gus	Hba	Hbb	IdH-1	Mod-1
STR/N (a,b,C)'	a	a	b	b	b	b	b	b	?	S	b	b
STR/1N (a,b,C,s)'	b	a	b	?	b	b	b	?	f	S	b	b

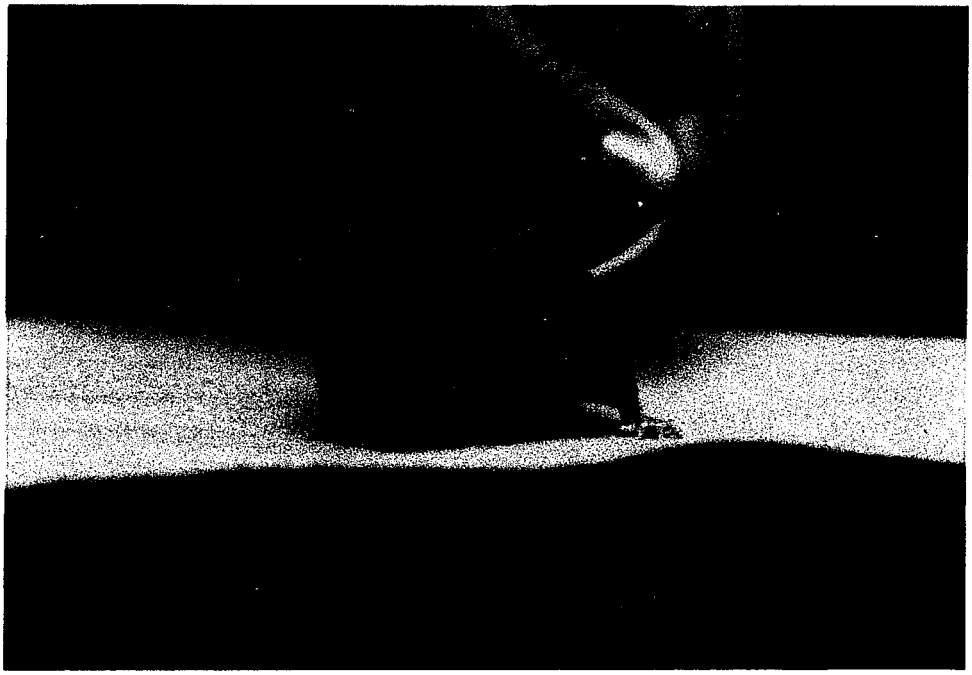
	4 major urinary protein		5 phosphoglucomutase	9 transferrin	2 hemolytic complement	19 lymphocyte antigen	9 thymus cell antigen
	Mup-1	Pep-3	Pgm-1	Trf	Hc	Lyt-1	Thy-1
STR/N	a	b	b	a	1	?	?
STR/1N	a	?	b	?	1	b	a

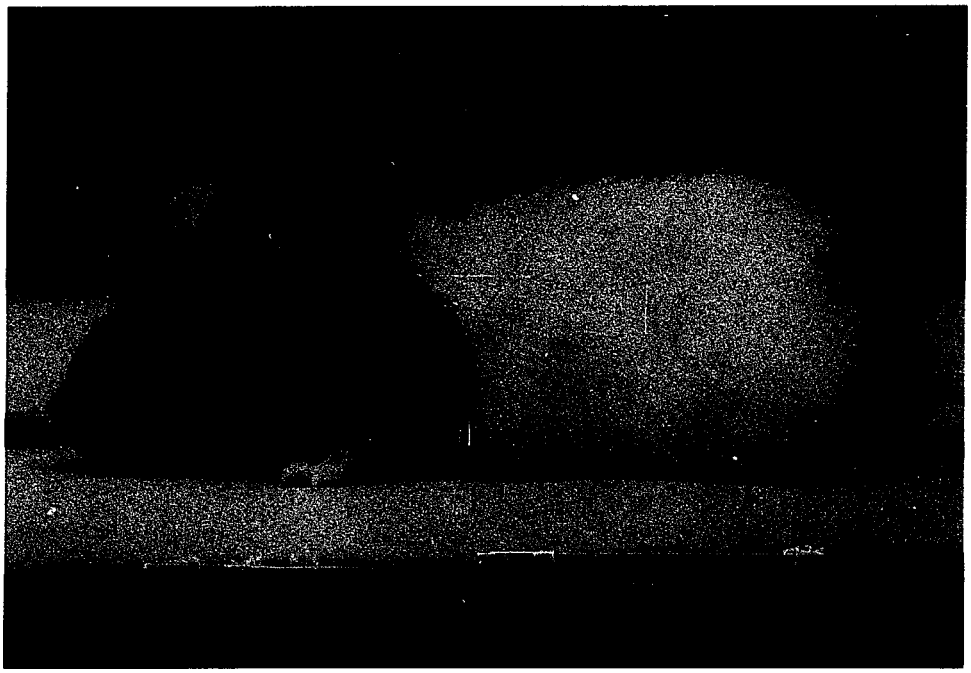
---

[1] Albino locus, C, chromosome 7; non-agouti locus, a, chromosome 2; piebald locus, S, chromosome 14; brown locus, b, chromosome 4.

* Chromosome number.

Figure 6 Photograph of STR/N and STR/1N mice. The STR/1N (bottom panel) mouse is a color mutant of STR/N (top panel), but is closely related genetically.





The STR/1N mice have been studied with respect to many characteristics (Table 4). The degenerative joint disease is genetically governed and multiple genes are involved. These genes have an overall recessive behavior without major sex linkage. Genetic dissociation between the joint disease, obesity, and hyperlipidemia has been demonstrated. The etiology of their osteoarthritis is unknown.

Leon Sokoloff (1956) characterized the pathology of the joint disease in STR/1N mice with regard to joint distribution and histologic changes in the knee joint. The knee joint is by far the area most severely affected. The lesions usually occur symmetrically, on both sides of the body. The medial condyles are affected to a much greater degree than the lateral. The other joints affected in the 1N mouse are the humeroulner, first carpometacarpal, second metatarsophalangeal, sacroiliac, thoracic vertebrae, and capital- and costovertebra.

Sokoloff's histologic analysis of the osteoarthritis in the 1N mouse defined a scale of increasing severity of the cartilage degeneration (Figure 7). Walton (1977a, b, c) has made an even more detailed histological analysis using an anterior-posterior plane of section rather than the sagittal plane. The disease process in Walton's STR/ORT strain, a sub-strain of the STR/1N, is initiated at the interface of the medial condyle and cruciate ligaments. Walton suggests that this might have some relevance to the etiology of the disease.

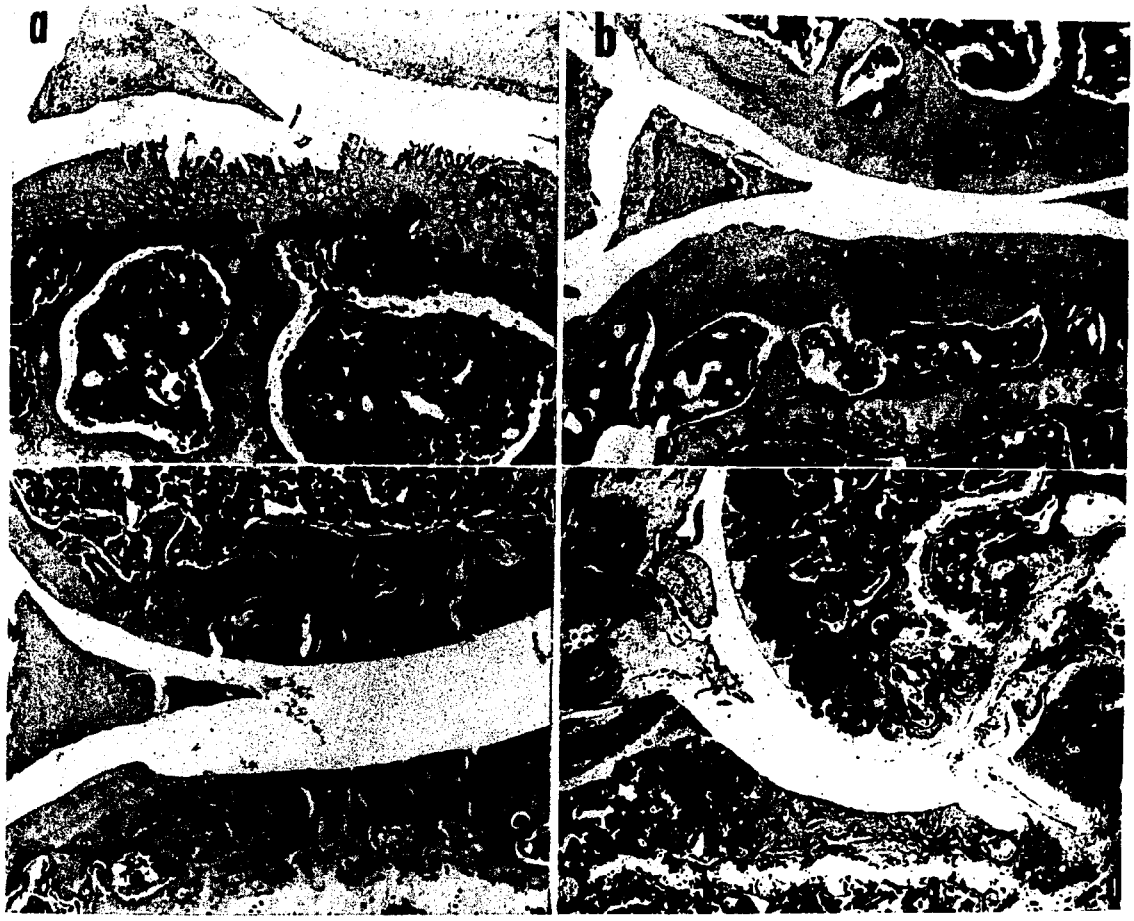
No disturbance in the gait has been recognized in these arthritic animals. The lesions appear at an age when the animals are relatively sedentary, whether they have arthritis or not. In the more advanced

TABLE 4

PARAMETERS STUDIED IN THE STR/1N MOUSE IN RELATION TO OSTEOARTHRITIS

PARAMETER	REFERENCE
body weight	Sokoloff, Mickelson, Silverstein, Jay, & Yamamoto, 1960; Walton, 1979
increased incidence of hepatomas	Sokoloff, 1956
anemia	Sokoloff, 1956
increased plasma lipid concentrations	Sokoloff, 1956
thyroid function; epiphyseal maturation	Sokoloff & Jay, 1956b; Silverstein, Sokoloff, Jay, & Mickelsen, 1960; Silverstein, Sokoloff, Mickelsen, & Jay, 1960
skeletal variation	Sokoloff, 1956
bone thickening	Walton & Elves, 1979
diet: fat content	Sokoloff & Mickelsen, 1965
response to sex hormones	Sokoloff, 1961; Sokoloff, Varney, & Scott, 1965
response to Rumalon	Silberberg, Silberberg, & Rüttner, 1964
genetics	Sokoloff, Crittenden, Yamamoto, & Jay, 1962
patellar displacement	Walton, 1979

Figure 7 Histologic grading of severity of the cartilage degeneration seen in the osteoarthritic mouse knee joints. (a) 1+ osteoarthritis; a small area of fibrillation and disruption of articular cartilage seen on tibial surface. (b) 2+ osteoarthritis; much of the articular cartilage on femoral and tibial surfaces is gone. (c) 3+ osteoarthritis; erosion has progressed into the epiphysis of the tibia. There is an increase of bone in the epiphyses. (d) 4+ osteoarthritis; a large concave defect is present in the epiphysis of the tibia. All sections stained with hematoxylin and eosin. (a)-(c) magnification x105, (d) magnification x45. (Sokoloff, 1956).


a
b

lesions the knee joint appears somewhat enlarged and the capsule is more opaque than normal and thickened. Sokoloff (1956) reported that "at times, the patella appears to be displaced medially" (p. 124). Walton (1977a, b) found a significant correlation between the osteoarthritis in his mouse strain and patellar dislocation. In a later study (Walton, 1979a), osteoarthritis of the medial condyle was prevented by surgical stabilization of the patella. Furthermore, surgical production of medial patellar dislocation in a non-arthritic-prone mouse strain resulted in OA of the medial condyle. Walton proposes that "the much higher incidence of the disease in STR/1N and STR/ ORT mice over all other strains is probably due to their predisposition for patella dislocation" (p. 171). But "no explanation is offered as to the cause of the patella dislocation in STR/ORT mice. The anatomy of the bones and quadriceps mechanisms were identical in both STR/ORT and CBA/ORT (control) strains, as far as could be ascertained visually" (p. 171). It may be that STR/1N mice have a predisposition (genetic) to developing osteoarthritis which is enhanced by their tendency for patellar displacement. Certainly OA occurs in some knee joints without patellar dislocation as well as in other joints. It would seem that degenerative joint disease in the STR/1N mouse has both a metabolic and mechanical element.

Biochemical Studies of Mouse Cartilage Proteoglycans

Several developmental abnormalities in mice are associated with cartilage disorders (Table 5). Brachymorphia, achondroplasia,

TABLE 5

DEVELOPMENTAL ABNORMALITIES OF MICE ASSOCIATED WITH CARTILAGE DISORDERS

	LEVEL OF CHARACTER-IZATION*	MATRIX COMPONENTS	REFERENCE
phocomelia (pc)	1	?**	Huecksohn-Waelsch, Hagedorn, & Siskin, 1956
shorthead (sho)	1	?	Fitch, 1961
achondrophasia (cn/cn)	1,2,3	normal PG	Konyukhov & Paschin, 1967, 1970; Lane & Dickie, 1968; Shepard, Fry, & Moffett, 1969; Bonucci, Del Marco, Nicoletti, Petrinelli, & Pozzi, 1976; Silberberg, Haeler, & Lesker, 1976; Kleinman, Pennypacker, & Brown, 1977
brachymorphia (bm/bm)	1,2,3	normal collagen undersulfated PG	Lane & Dickie, 1968; Orkin, Pratt, & Martin, 1976; Sugahara & Schwartz, 1979; Orkin, Williams, Cranley, Poppke, & Brown, 1977
stubby (stb/stb)	?	?	Lane & Dickie, 1968
cartilage anomaly (can/can)	1,2	normal collagen reduced PG	Johnson & Wise, 1971
cartilage matrix deficiency (cmd/cmd)	1,2,3	normal collagen defective core protein in PG	Rittenhouse, Dunn, Cookingham, Calo, Spielgelman, Dooher, & Bennett, 1978; Kimata, Barrach, Brown, & Pennypacker, 1981
brachypodism (bp/bp)	1	?	Grüneberg & Lee, 1973
chondrodysplasia (cho/cho)	1,2,3	normal collagen normal PG	Seegmiller, Fraser, & Sheldon, 1971; Seegmiller, Myers, Dorfman, & Horwitz, 1981

* 1 = histology, 2 = EM ultrastructure, 3 = biochemistry

** ? = not studied

cartilage matrix deficiency, and chondrodysplasia have been characterized biochemically. Achondroplastic (cn/cn) mice have normal cartilage matrix components, but are so severely affected that homozygotes die at 1-3 months of age. Brachymorphic (bm/bm) animals are similar to achondroplastic mice in growth characteristics and gross features but are less severely affected. The cartilage proteoglycans from bm/bm animals have been found to be undersulfated as a result of defective PAPS synthesis. Chondrodysplasic (cho/cho) mice are severely affected and die soon after birth, but their cartilage proteoglycans appear to be of normal size, aggregability, and sulfation. The cartilage matrix deficiency (cmd/cmd) mice are indeed deficient in matrix and also die soon after birth. The decreased amount of matrix has been shown to be the result of decreased synthesis of proteoglycan which in turn is due to decreased synthesis of the core protein. Thus, of the four disorders that have been characterized biochemically, two (cn/cn and cho/ cho) have normal collagen and proteoglycan but are severely affected, one (cmd/cmd) has abnormal proteoglycan synthesis and is severely affected, and one (bm/bm) has abnormal cartilage proteoglycans but the condition is compatible with life. The osteoarthritis of the STR/1N mouse is compatible with life despite the extensive loss of cartilage seen in the knee joints. Although knee joints of these animals have been studied histologically and by electron microscopy, the articular cartilage proteoglycans have not been isolated and characterized biochemically.

Purpose of Study

The major purpose of the present study was to re-evaluate the status of proteoglycans in osteoarthritic articular cartilage, exercising maximum caution in the isolation and fractionation of the PG in order to avoid the pitfalls experienced by others. The STR/1N mouse model offered many advantages: (1) the disease develops spontaneously, (2) the incidence is high, 100% in males, 80% in females, (3) many animals can be studied with minimal individual variation, (4) age-matched control animals are readily available.

The small size of the mouse knee joint dictated the development of micromethods of extraction and isolation of the articular cartilage proteoglycans. Furthermore, it was important to minimize proteolysis during the isolation procedure. Especially critical was the rapid separation of extracted proteoglycans from proteins including proteases. In the present work, a methodology was developed which ensured minimal proteoglycan degradation by using rapid low temperature extraction, isolation in the presence of protease inhibitors, and preformed CsCl density gradients in the Beckman Airfuge. The Airfuge technique has been published (Rostand, Baker, Caterson, & Christner, 1982).

METHODS

Materials

Cesium chloride (Analar grade) was purchased from Gallard-Schlesinger Chemical Manufacturing Corporation. Sepharose CL-2B and CL-6B were from Pharmacia Fine Chemicals. Guanidine hydrochloride (grade I), iodoacetic acid, phenylmethanesulfurylfluoride, and papain were purchased from Sigma. The papain was recrystallized by a published procedure (Kimmel, 1954). Benzamidine hydrochloride, 6-aminohexanoic acid, and x-ray film (BB-1) were from Eastman. Streptomyces hyalurolyticus hyaluronidase was obtained from Calbiochem-Behring. Chondroitinase AC-II, ΔDi-4S, ΔDi-6S, and ΔDi-OS were from Miles Laboratories. Carrier-free ^{35}S-sulfate was from New England Nuclear and carrier free ^{125}I, in NaOH solution, was from Amersham Corp. Scintiverse I was from Fisher Chemical Company. Biogel P-2 and "Enzymobeads" were from Bio-Rad. Controlled-pore glass (CPG) DEAE Glycophase (200 Å pore diameter, 74-125 μ particle size) was purchased from Pierce Chemical Company. Heat-killed, formalin-treated Staphylococcus aureus was prepared according to Goding (1978). A specific antiserum was obtained from a rabbit immunized with BNC link proteins 1 and 2 and has been characterized (Caterson, Baker, Levitt, & Paslay, 1980; Caterson, Baker, & Christner, 1980). All other reagents were of analytical reagent grade.

Proteoglycan Fractions

Proteoglycans were extracted from bovine nasal cartilage (50 g) in 500 ml of 4 M guanidine hydrochloride, 0.05 M sodium acetate, pH 6.5, and protease inhibitors, 5 mM benzamidine hydrochloride, 100 mM 6-aminohexanoic acid, 1 mM iodoacetate, and 1 mM phenylmethanesulfonylfluoride (Oegema, Brown, & Dziewiatkowski, 1977). Extraction was done with stirring, at 4°C, for 24 h. Proteoglycan aggregate (A1), proteoglycan monomer (A1D1), and the link protein-rich fraction (A1D4) were subsequently isolated from this extract as described previously (Baker & Caterson, 1979).

Similarly, proteoglycans were extracted from rat chondrosarcoma, and the aggregate fraction (A1) was isolated (Caterson & Baker, 1979).

Analytical Methods

Hexuronic acid determinations were done either manually according to the method of Bitter and Muir (1962) or by an automated procedure (Ford & Baker, 1978). Hyaluronic acid was determined according to the method of Jourdian, Wolfman, Sraber, & Distler (1979) which employs the specific hyaluronidase from S. hyalurolyticus. The method of radioimmunoassay used to quantitate the link proteins from bovine nasal cartilage aggregate has been described elsewhere (Caterson et al., 1980). The antiserum used is specific for BNC link proteins 1 and 2 (Caterson et al., 1980; Caterson et al., 1980) and cross-reacts with link proteins from other species (Caterson et al., 1980).

For assay of radioactivity, aqueous samples were diluted to 1.0 ml with deionized water, Scintiverse added (2.5 ml), and the resulting gel counted in a Packard Tricarb Liquid Scintillation Spectrometer.

Analysis of sulfate in glycosaminoglycans was performed by the barium-rhodizonate method of Terho and Hartiala (1971) as modified by Silvestri, Hurst, Simpson, and Settine (1982). The major modification was the liberation of the sulfate from the glycosaminoglycans by a brief pyrolysis rather than acid hydrolysis. Potassium sulfate was used as the standard.

Polyacrylamide Gel Electrophoresis

Analysis of fractions by polyacrylamide gel electrophoresis in SDS was carried out on a Hoefer vertical slab gel apparatus (model SE 500) on 11.1% gels (1.5 mm), using a discontinuous buffer system as described by Neville (1971). The gels were either fixed in 40% methanol-7.5% acetic acid and stained by Coomassie blue R or dried at room temperature under vacuum and subjected to autoradiography for 24 h. at -70°C.

Gel Chromatography of Proteoglycans

Proteoglycan samples (80-800 µg of proteoglycan in 200-300 µl) were applied to a column (0.5 x 100 cm) of Sepharose CL-2B and eluted with 0.15 M sodium sulfate at a flow rate of 1 ml/h. The eluant was

monitored continuously at 206 nm using a UVicord S (LKB, model 2138) and 2-channel recorder (LKB, model 2210). Fractions (0.4 ml) were collected for assay of uronic acid and radioactivity. Columns eluted with 4 M guanidine hydrochloride could not be monitored at 206 nm due to the absorbance of the guanidine. Fractions containing 4 M guanidine hydrochloride were assayed for uronic acid manually.

Molecular Weight Determination of Bovine Nasal Cartilage Proteoglycan Monomer (BNC A1D1)

A solution of BNC A1D1 (1.0 ml of ~4 mg/ml in 0.14 M Na_2SO_4) was dialyzed exhaustively against 0.1 M NaCl-0.12 mM $NaHCO_3$, pH 6.8. The uronic acid concentration after dialysis was determined and assuming a uronic acid content of 25% of the dry weight, the concentration of monomer was calculated to be approximately 3.2 mg/ml. Aliquots of the dialyzate were used in the solvent channel of the centrifuge cell.

A Spinco model E ultracentrifuge equipped with Rayleigh interference optics and a temperature control unit was used. Photographs of interference patterns were taken using Kodak Spectroscopic II-G plates. An AN-J rotor was used. Cells were equipped with sapphire windows and with 30-mm standard, double sector, aluminum-filled epoxy resin center pieces. The solution channel was filled with 0.03 ml of fluorocarbon FC-43 base fluid and 0.25 ml of solution. The solvent channel was filled with a volume of solvent 0.05 ml greater than the volume of solution. The solution was diluted to approximately 0.2 mg/ml proteoglycan and the speed was 4,800 RPM. The interference

pattern photographed at 24 h was used for calculating the molecular weight according to the formula:

$$M_{app.} = \frac{\text{slope} \times 2RT}{w^2(1-\bar{v}\rho)}$$

Slope taken from plot of $\log (y_r - y_o)$ vs. r^2.

$\bar{v}$ = 0.545 ml/gm
R = 8.313×10^7 ergs mol^{-1} K^{-1}
T = 293A
W = 2Π rev/sec.
$y_r - y_o$ = fringe displacement
v° = partial specific volume of proteoglycan (Hopwood & Robinson, 1975)

Velocity runs were made at 56,000 RPM in an AN-H rotor. Svedberg values were calculated for two different concentrations of proteoglycan using the formula:

$$S^{\circ}_{20,w} = \frac{1}{w^2 r}\frac{dr}{dt} = \frac{2.303}{60\ w^2}\frac{d \log x}{dt'}$$

x = distance from center of rotation (cm)
t = time (sec)
w = angular velocity (radians/sec)

Radioiodination

After dialysis to associative conditions, an aliquot (50 µl) of the 4 M guanidine hydrochloride extract from bovine nasal cartilage was iodinated with ^{125}I-iodine (1 mCi) using immobilized glucose oxidase and lactoperoxidase ("Enzymobeads") as described by Christner, Caterson, and Baker (1980). A portion of this iodinated extract,

equal to approximately 100,000 cpm (2 µl), was mixed with 300 µl of the unlabeled extract and fractionated on the Airfuge under "associative" conditions to obtain the iodinated A1, A2, A3 fractions.

For analysis of endogenous proteoglycans from mouse articular cartilage an aliquot (50-100 µl) of an A1 fraction was iodinated by the chloramine-T method (Sonada & Schlamowitz, 1970) for 2 min at room temperature with 1 mCi of [^{125}I]iodine. After stopping the iodination reaction, carrier BNC A1 (200 µg) was added to diminish nonspecific absorption of mouse ^{125}I-proteoglycan during subsequent gel chromatography. Similarly, for analysis of endogeneous proteoglycan monomers from mouse articular cartilage, a D1 fraction was isolated from a 4 M guanidine hydrochloride extract and an aliquot (100 µl) was iodinated with [^{125}I]iodine (1 mCi) by chloramine-T for 2 min at room temperature. Carrier BNC A1D1 (400 µg) was added after stopping the iodination reaction. Aliquots (10-20 µl, ~50,000 cpm) of both ^{125}I-A1 and ^{125}I-D1 were mixed with carrier BNC A1 and BNC A1D1, respectively, for analysis by gel chromatography.

Conditions for Using the Beckman Airfuge

The Airfuge was operated according to the general procedure described in the Beckman manual. Centrifugations were carried out in a cold room at 4°C following the Beckman recommendations for cold room installation. In addition, the length of copper tubing used for the cooling coil was increased from 10 feet to 20 feet and was immersed in

a salt-ice bath at -15°C. With this arrangement, the rotor temperature could be maintained at 5-10°C for the 4-h runs. The A-100/30 rotor was preferred because of its capacity for 240-µl tubes, although for most runs, thick walled slightly tapered polyethylene tubes of 100 µl capacity were used. These tubes (Brinkman Instruments micro test tubes, catalog no. 22-36-440-5) were cut to size (2 cm external length). Bothwell, Howlett, and Schachman (1978) suggest that the narrow bore and tapered shape of these tubes may facilitate the transport of macromolecules to the bottom of the tube and minimize convective stirring during deceleration of the rotor. Beckman polyallomer tubes (240 µl) were also found to be satisfactory, but the polycarbonate tubes (240 µl) supplied with the centrifuge did not withstand the 4-h high speed runs. All centrifugations were run at maximum air pressure of 30 p.s.i. which produces a centrifugal force at the bottom of the tubes of approximately 160,000 x g. Braking was accomplished with an adjusted automatically timed delay of 3 min (setting 4.5) between turning off the air supply to the driving jets and the application of the mechanical brake.

Maintenance and Breeding of Mice

Breeding pairs of STR/1N and STR/N inbred strains were obtained from the National Institutes of Health, Genetics Research Unit. The animals were maintained in a room exclusive of other animals at the animal services facilities of the University of Alabama in Birmingham Medical Center. No more than six adult animals were housed in each

cage (8" W x 12" D x 5" H), fewer if pups were present. Disposable filter tops were used on all cages. The diet consisted of Wayne Mouse Breeder Blox which is 20% protein and 10% fat. Water was given ad libitum.

The semi-barrier conditions for housing the animals were as follows:

1. 2 sets of boxes: 1 in use
   1 clean — Boxes changed twice/week

   2 sets of bottles: 1 in use — H_2O bottles changed once/week
   1 clean

2. Mice were handled first thing in morning, before contacting any other animals. Gloves were worn.

3. Filter tops were used on cages. One top removed at a time when changing.

4. Mainly two people handled the mice, with 1 in reserve.

5. Door was flush with floor, with no cracks to allow outside rodents in. The door was kept locked.

Cages and water bottles were kept separate from others at the animal facility as were the feed and bedding. The room was temperature-controlled (70-75°F) with a 12-h. light-dark cycle.

Histology of Mouse Knee Joints

Intact knees were skinned and excess tissue removed without disrupting the joint. The whole joint was fixed in 10% neutral buffered formalin for 24 h. After fixation, the joints were decalcified, sectioned, and stained by the standard procedure in use in the Histology Laboratory, Department of Pathology at UAB. In general, this process involves decalcification at room temperature in formic acid buffered by sodium citrate. Decalcification required 7-10 days for the mouse knee specimens. The decalcified joints were then washed in water for 24 h., dehydrated and set in paraffin blocks. Serial sagittal sections through the knee joint (6 microns thick, 20-25 microns apart) were taken and stained with hematoxylin and eosin. A series of 12 sections was usually sufficient to view both condyles of the joint.

Scanning Electron Microscopy of Joint Surfaces

The mice were killed by cervical dislocation and the hind limbs amputated at the mid-femoral shaft. The knee joints were carefully disarticulated with special care being taken to ensure the menisci were removed from the medial condyles without damaging the articular cartilage. The femur and tibia were washed in saline to remove blood cells and tissue debris, then fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.35, for 2 h. The specimens were rinsed overnight in cacodylate buffer, dehydrated in graded alcohols and critical point dried. The bone shafts were trimmed to give approximately 5 mm

of the distal end of the femur and slightly less of the proximal end of the tibia. The cut ends of the shafts were mounted and coated with gold-palladium in a Hitachi HUS-4 vacuum evaporator. The articular surfaces were viewed using a Cambridge Mark 2A scanning electron microscope at an accelerating voltage of 20 kV.

In Vivo Incorporation of ^{35}S-Sulfate into Mouse Articular Cartilage Proteoglycans

Each mouse was injected intraperitoneally with 1 mCi carrier-free ^{35}S-sulfate in 0.5-1.0 ml sterile 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2. The animal was sacrificed by cervical dislocation 18-20 h. after injection. The hind knee joints were removed from the animal, opened as quickly as possible, and the femoral and tibial condyles exposed. The articular cartilage was cut from the condyles with a scalpel blade while viewed under a dissecting microscope. The microscope platform was ice-cooled. The ^{35}S-labelled cartilage slices were used either for proteoglycan extraction or glycosaminoglycan characterization.

Extraction of ^{35}S-Proteoglycans from Articular Cartilage

Cartilage (wet weight, ~ 3 mg/mouse) from either a single mouse or pooled from several animals was extracted for 5 h with stirring in 0.4 M or 4 M GuHCl (500 µl), containing protease inhibitors and carrier BNC A1 (1 mg/ml), at -5°C (0.4 M extraction) or -10°C (4 M extraction). Proteoglycan aggregates were reconstituted from the 4 M

extract by dialysis for 8 h against 1.56 CsCl containing 0.15 M Na_2SO_4 and protease inhibitors (d = 1.2 g/ml) at -5°C. The dialyzed extract was layered on preformed CsCl density gradients. After centrifugation for 4 h in the Airfuge, the A1, A2, and A3 fractions were obtained. Associative extracts (0.4 M GuHCl) were dialyzed similarly to get rid of free ^{35}S-sulfate or in some cases were put directly onto associative CsCl gradients. An aliquot (5,000-10,000 cpm) of the ^{35}S-A1 was chromatographed on Sepharose CL-2B.

In order to isolate PG monomer, the mouse articular cartilage was extracted with 4 M GuHCl as described above. CsCl (263 mg/ml) was added, and the extract layered on preformed dissociative CsCl density gradients and centrifuged for 4 h in the Airfuge. The D1, D2, and D3 fractions were obtained. An aliquot (5,000-10,000 cpm) of the ^{35}S-D1, containing the carrier BNC PG, was chromatographed on Sepharose CL-2B.

Isolation and Characterization of ^{35}S-Labelled Glycosaminoglycan from Mouse Articular Cartilage

Cartilage slices from each ^{35}S-injected mouse (two hind knee joints) were incubated for 18-24 h at 65°C in 0.5 ml of 0.1 M sodium acetate, 5 mM EDTA, 3 mg/ml cysteine HCl, pH 6.2, and papain (2x recrystallized), 0.5 mg/ml. At the end of this incubation period, carrier BNC CS (200 µg) was added and the mixture applied directly to a Pharmacia PD-10 column. The PD-10 column was pre-equilibrated and eluted with 0.1 M Tris acetate pH 7.3 (chondroitinase buffer). The ^{35}S-macromolecular material eluting at V_o was pooled and used either

for analysis on Sepharose CL-6B and CPG-DEAE or for chondroitinase digestion.

Gel Chromatography of ^{35}S-Glycosaminoglycans

The ^{35}S-glycosaminoglycans (~5,000 cpm per 200-300 µl) eluting in the V_o of the Pharmacia PD-10 column were applied to a column (0.5 x 100 cm) of Sepharose CL-6B and eluted with 0.2 M pyridine acetate, pH 5 at a flow rate of 1.2 ml/hr. Fractions (0.4 ml) were collected for assay of uronic acid and radioactivity.

Ion-Exchange Chromatography of ^{35}S-Glycosaminoglycans

A mixture of ^{35}S-glycosaminoglycans and carrier BNC GAG (0.5 ml containing ~10,000 cpm and ~100 µg uronic acid, respectively) was applied to a column (1.0 x 10 cm) of CPG-DEAE. The column had been equilibrated with 0.05 M Tris-HCl, pH 7.2 and was eluted with a gradient from 0-1 M NaCl in the same buffer. The total gradient volume was 300 ml. Fractions (3 ml) were collected and analyzed for UA, cpm, and conductivity.

Chondroitinase Digestion of ^{35}S-Glycosaminoglycans

Chondroitinase ACII or ABC (0.1 units/ml) was incubated with the mixture of ^{35}S-glycosaminoglycans and carrier BNC glycosaminoglycans (0.5-1.0 ml) from the PD-10 column for 3-5 h at room temperature. The extent of digestion was followed by monitoring the absorbance at 232 nm (Saito et al., 1968). After digestion was complete, the sample was applied directly to a column (1 x 25 cm) of Biogel P-4 and eluted

with 0.2 M pyridine acetate, pH 5. Fractions (0.5 ml) were collected for assay of radioactivity. The ^{35}S-labelled unsaturated disaccharides eluting near V_T were pooled, lyophilized, and finally analyzed by high pressure liquid chromatography or thin-layer chromatography for their isomeric content.

High Pressure Liquid Chromatography of Disaccharides from Chondroitinase Digestion of Glycosaminoglycans

Disaccharides obtained from chondroitinase digestion of CS chains can be resolved on a weak anion-exchange resin in a high pressure liquid chromatography system according to the method of Hjerpe, Antonopoulos, & Engfeldt (1979). The disaccharide peak from the Biogel P-2 column was pooled, lyophilized, and then redissolved in a minimum amount (usually 50-100 µl) of elution buffer, 0.02 M sodium sulfate, 0.01 M sodium acetate pH 5.5 or 0.2 M sodium acetate pH 5.5. Injection volumes were 20 µl.

Ratios of ΔDi-4-S to ΔDi-6-S isomers were determined by weighing cut-out peaks from either 232 nm absorbance or ^{35}S-sulfate profiles.

A Laboratory Data Control HPLC pump (ConstaMetric III) and monitor (SpectroMonitor III) were used. The column was a Waters Carbohydrate Column from Waters Associates, Inc., Milford, Massachusetts.

Thin-Layer Chromatography of Disaccharides from Chondroitinase Digestion of Glycosaminoglycans

The disaccharides resulting from digestion of GAG with chondroitinases were separated on microcellulose TLC plates according to the

system described by Wasserman, Ber, and Allalouf (1977). This process involves a desalting step in which the spotted plates are first developed in 1-butanol-ethanol-water (52:32:16) for 6 h. After drying in air the plates are developed for 6 h in 1-butanol-acetic acid-1N ammonia solution (2:3:1). The unsaturated disaccharides from the carrier GAGs or standards are visible under shortwave UV light. For quantitation of ^{35}S-labelled material the plates were divided into 0.5 cm segments, scraped-off, and counted directly after addition of water (1 ml) and Scintiverse (2.5 ml).

Extraction and Isolation of Endogenous Proteoglycans from Mouse Articular Cartilage

Articular cartilage from 3-6 mice was obtained as described for ^{35}S-proteoglycans. Extraction and isolation was achieved in the same manner as well, but carrier proteoglycan was omitted from the extraction mixture. The A1 fraction obtained was radiolabelled with ^{125}I-iodine as described under "Radioiodination." An aliquot (10-20 µl, ~50,000 cpm) of the ^{125}I-A1 was mixed with unlabelled A1 from rat chondrosarcoma (200 µl, 200 µg of PG in 0.15 M Na_2SO_4) and chromatographed on Sepharose CL-2B. The D1 fraction obtained was radiolabelled with ^{125}I-iodine (see "Radioiodination"). An aliquot of the ^{125}I-D1 was mixed with unlabelled A1D1 from BNC and chromatographed on Sepharose CL-2B.

RESULTS AND DISCUSSION

Airfuge Fractionation

There have been several reports which indicate the feasibility of using the Beckman Airfuge to determine molecular weights of proteins on the basis of their sedimentation properties (Bothwell, Howlett, & Schachman, 1978; Pollet, Maase, & Standaest, 1979). Our need to isolate and fractionate PG on a small scale, i.e., from mouse articular cartilage, prompted an investigation of the usefulness of the Airfuge for this purpose. It was important to establish conditions for separation of proteoglycan aggregate from other proteins in cartilage extracts and for the fractionation of the aggregate into its components (PG monomer, HA, and link proteins).

Initial experiments indicated that a satisfactory density gradient (1.3-1.6 g/ml) could not be formed by centrifuging a CsCl solution of uniform density at 30 p.s.i. for as long as 18 h. Therefore, preformed CsCl gradients were made by layering successively into each tube solutions of density 1.65 g/ml (30 µl), 1.40 g/ml (30 µl), and the sample dissolved in a CsCl solution of density 1.20 g/ml (40 µl). Preformed CsCl gradients in 4 M guanidine hydrochloride were similarly prepared for "dissociative" fractionation of the proteoglycan aggregate components. After centrifugation (see "Methods" for conditions),

the contents of each tube were removed as 3 approximately equal aliquots by aspiration from the meniscus using a Hamilton syringe. Densities of pooled fractions were measured pycnometrically using a 50-µl constriction pipette.

To determine whether proteoglycan aggregates could be sedimented to the bottom of the CsCl gradient described, an A1 preparation from bovine nasal cartilage (4 mg/ml) was centrifuged under associative conditions for 4 h at maximum speed. The three fractions obtained, A1, A2, and A3 (bottom to top), were analyzed for uronic acid. The results show that 97% of the proteoglycan (measured as uronic acid) accumulates in the bottom fraction, A1 (Table 6). Thus, although the

TABLE 6

ISOLATION OF PROTEOGLYCAN AGGREGATES AND THEIR COMPONENTS USING PREFORMED CsCl DENSITY GRADIENTS IN THE BECKMAN AIRFUGE

Fractions	Density		Uronic Acid		Hyaluronate	Link Proteins
	Initial	Final				
	g/ml		ug/ml	%	%	
A3	1.35	1.42	16	1		
A2	1.46	1.43	24	2		
A1	1.67	1.47	1600	97		
A1D3	1.35	1.43	72	3	83	95
A1D2	1.46	1.48	304	11	17	5
A1D1	1.69	1.51	2340	86	0	0

preformed gradient shows considerable change after centrifugation, the proteoglycan aggregate migrates from the top and concentrates in the bottom fraction. An aliquot of the A1 fraction was chromatographed on Sepharose CL-2B as described in "Methods" and the elution profile, as determined by absorbance at 206 nm and by uronic acid assay, shows that most of the proteoglycan is eluted at V_0 (Figure 8a). Therefore, aggregation of the proteoglycans appears unaffected by the centrifugation procedure.

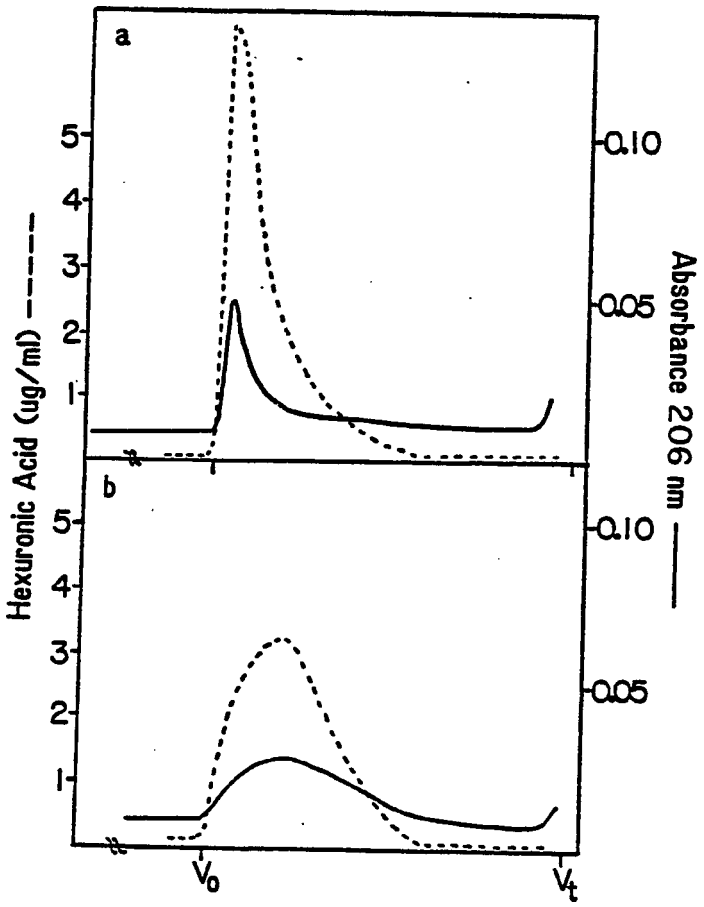
An important step in the isolation of proteoglycans is their separation from proteins, especially degradative enzymes, in the extract. To determine if this separation was achieved in the preformed gradients, an extract of bovine nasal cartilage in 4 M guanidine hydrochloride was prepared, dialyzed to associative conditions, and centrifuged in the Airfuge. The A1, A2, and A3 fractions were analyzed for protein (absorbance at 280 nm) and uronic acid (Table 7).

TABLE 7

SEPARATION OF PROTEOGLYCAN FROM OTHER PROTEINS IN A CARTILAGE EXTRACT

Fractions	Bovine Nasal Cartilage			
	Uronic Acid		Protein	
	ug/ml	%	A_{280}	%
A3	8	4	0.097	34
A2	6	3	0.090	26
A1	181	93	0.135	39

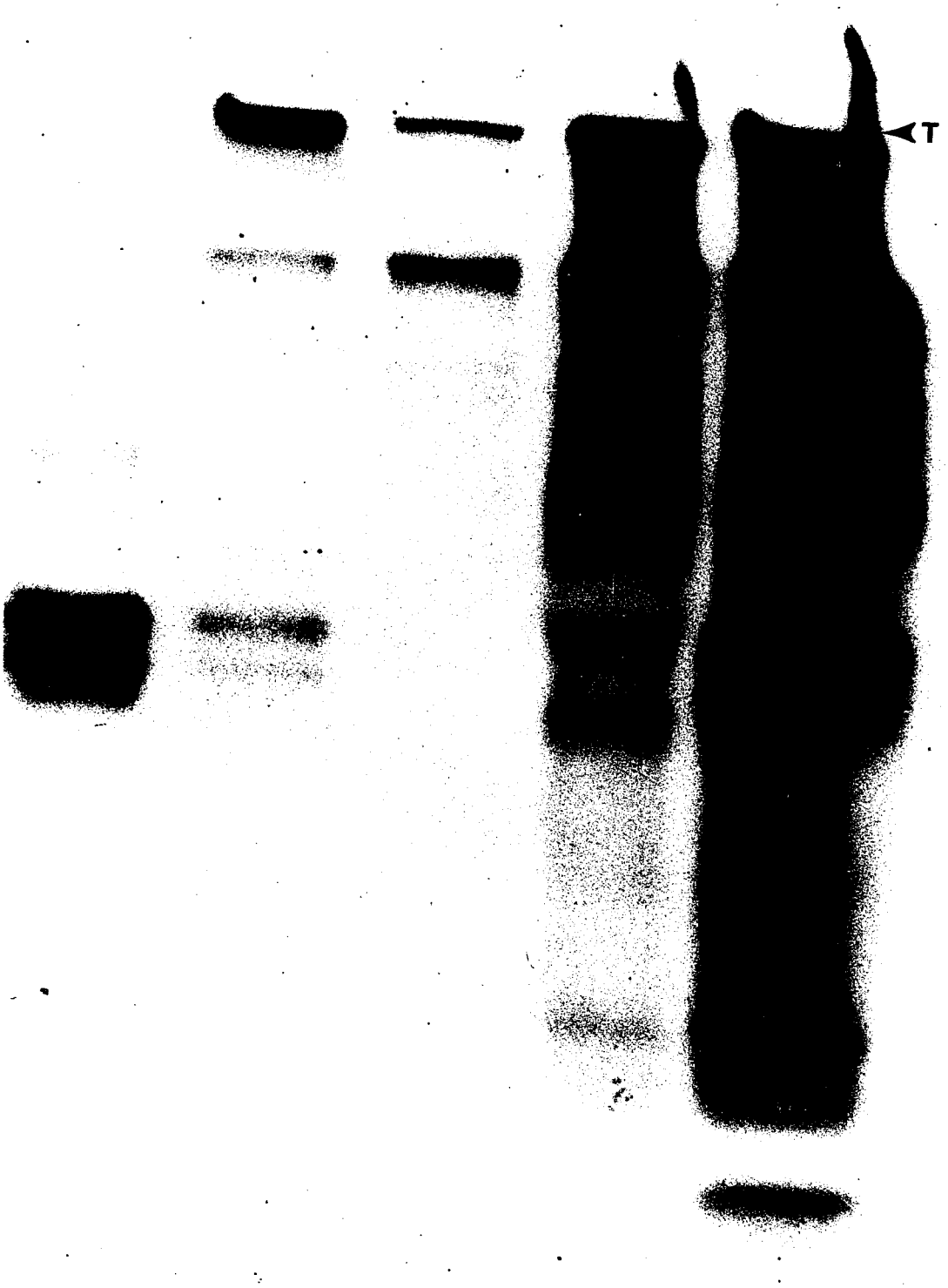
Figure 8 Sepharose CL-2B chromatography of bovine nasal cartilage proteoglycan fractions isolated on preformed CsCl density gradients in the Beckman Airfuge. Gradient fractions were diluted with 0.15 M Na_2SO_4 before application to the column (0.5 x 100 cm). Elution was carried out with 0.15 M Na_2SO_4 at 1 ml/h. The effluent was monitored continuously for absorbance at 206 nm, and fractions of 0.40 ml were collected for uronic acid assay. a, proteoglycan aggregate. A1 (~ 130 µg) in 0.15 M Na_2SO_4 (200 µl); b, proteoglycan monomer. A1D1 (~ 500 µg) in 0.15 M Na_2SO_4 (200 µl).


a
b
5
4
3
2
1
0.10
0.05
Hexuronic Acid (ug/ml) ----
Absorbance 206 nm ——
V_0
V_t

The absorbance at 280 nm indicates that some protein remains at the top of the gradient (i.e., in fraction A3). The majority of the uronic acid migrates to the bottom fraction, A1. Whether all the protein measured in A1 is an integral part of the proteoglycan aggregate, i.e., core protein and link(s), was determined in the following experiment. A ^{125}I-labeled extract of bovine nasal cartilage was centrifuged on a preformed "associative" CsCl gradient, and the A1, A2, and A3 fractions were recovered (see under "Methods"). Fractions were dialyzed against deionized water, freeze dried, and redissolved in sample buffer for separation by polyacrylamide gel electrophoresis in SDS. ^{125}I-labeled components were localized by autoradiography (Figure 9). The A1 fraction appears to contain only proteoglycan (at the surface of the stacker and running gel) and link proteins (the two closely migrating bands in the running gel). All other ^{125}I-labeled proteins are found in the A3 fraction. Therefore, it is evident that proteoglycan and protein extracted by 4 M guanidine hydrochloride from bovine nasal cartilage can be rapidly fractionated under associative conditions in the Airfuge into proteoglycan aggregate (A1) and protein (A3).

The efficiency of the Airfuge fractionation in separating proteoglycan from proteinases was further demonstrated by the following experiment. A solution of BNC A1,2 (4 mg/ml in 0.15 NaAc, pH 6.3, 1.56 M CsCl, d = 1.2 gm/ml) was treated in three different ways: A. incubated at 4°C for 4 h. B. mixed with trypsin at an enzyme:PG ratio of 1:500 (w/w) and incubated at 4°C for 4 h. C. mixed with trypsin at the same ratio as above, immediately layered onto preformed

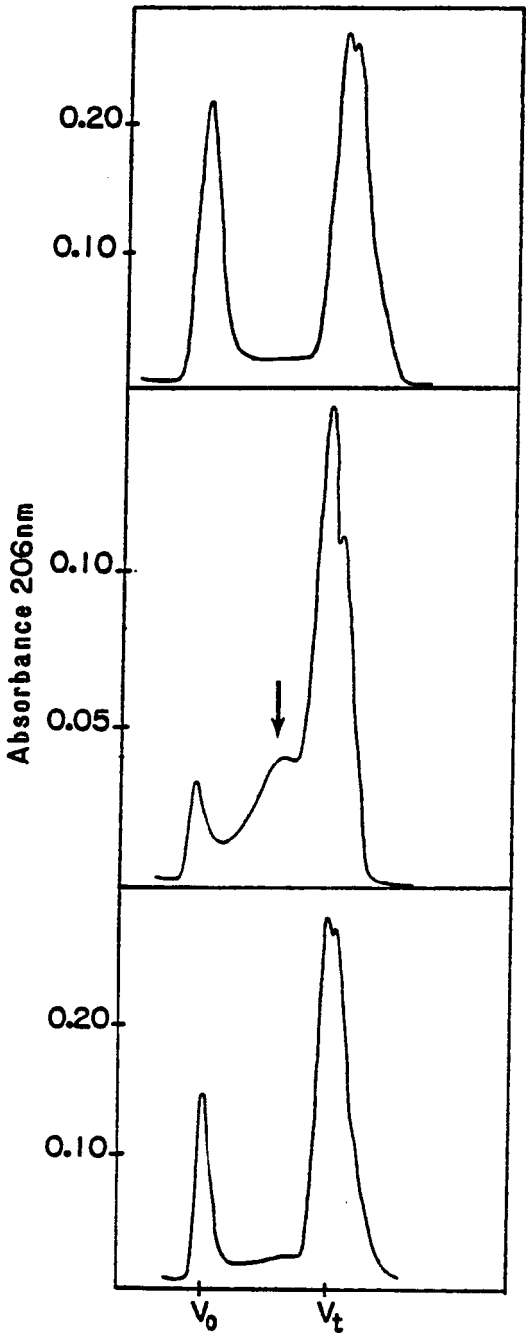
Figure 9 Autoradiogram of ^{125}I-labeled bovine nasal cartilage extract and Airfuge fractions after polyacrylamide gel electrophoresis in SDS. Fractions were dialyzed against deionized water, lyophilized, and redissolved in sample buffer for electrophoresis. Approximately 20,000 cpm in 100 µl were applied to each well of the 11.1% gel. a, BNC link proteins; b, c, and d, associative gradient fractions A1, A2, and A3, respectively, from ^{125}I-BNC; e, ^{125}I-BNC extract.


T
a
b
c
d
e

CsCl density gradients and centrifuged for 4 h in the Airfuge; the A1 fraction was isolated. An aliquot from each of the three solutions was chromatographed on a CPG 2500 column (0.5 x 100 cm) to determine the relative degradation of the proteoglycan as measured by the amount of excluded and included material (Figure 10). The absence of an included peak in the elution profile from solution A (4°C, 4 h, no enzyme) indicates no degradation has occurred under these conditions in the absence of trypsin. The large peak seen at V_T is due to the strong absorbance of acetate and CsCl and is seen for all three solutions. Incubation with trypsin for 4 h at 4°C (solution B) results in significant degradation as seen by a decrease in size of the excluded peak and a concomitant increase in included material (Figure 10B). The included material (indicated by arrow) is eluted at a position consistent with the known size of tryptic fragments from BNC PG. However, the immediate fractionation of the trypsin:PG mixture in the Airfuge (solution C) appears to have rescued the aggregate from degradation by the protease; included material is barely discernible (Figure 10C).

To determine whether the components of proteoglycan aggregate could be fractionated on preformed CsCl density gradients in the Airfuge, an A1 preparation from bovine nasal cartilage (4 mg/ml) was dissolved in 4 M guanidine hydrochloride, allowed to dissociate (24 h, 4°C), and centrifuged under "dissociative" conditions. The three fractions obtained, A1D1, A1D2, and A1D3 (bottom to top), were dialyzed, freeze dried, and analyzed for hyaluronic acid, link protein, and uronic acid (Table 6). The A1D1 fraction contained 86% of the

Figure 10 CPG-2500 chromatography of trypsin treated BNC A1,2 with and without fractionation on the Airfuge. The column (0.5 x 100 cm) was eluted with 0.15 M Na_2SO_4, pH ~ 7 at a flow rate of 20 ml/h. A) BNC A1,2, 4 mg/ml, 4 h at 4°C. B) trypsin + BNC A1,2 (1:500), 4 h at 4°C. C) trypsin + BNC A1,2 (1:500), Airfuge A1.


0.20
0.10
0.10
0.05
Absorbance 206nm
0.20
0.10
V_0
V_t

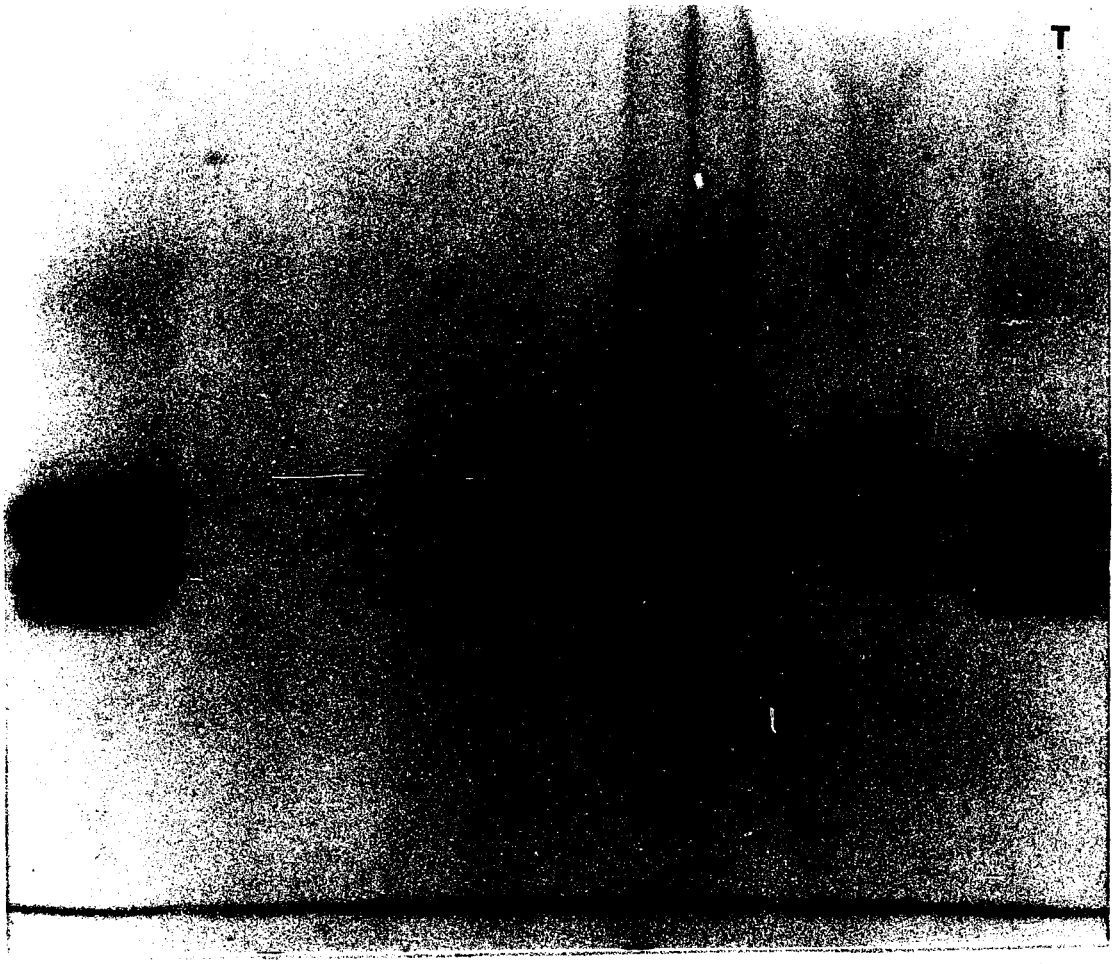
uronic acid, with 11% and 3% in the A1D2 and A1D3, respectively. This distribution is similar to that obtained by a conventional dissociative equilibrium density centrifugation (Oegema et al., 1977; Baker & Caterson, 1979). Chromatography of the A1D1 fraction on Sepharose CL-2B gave the broad included profile (K_{av} = 0.18), which is typical of bovine nasal cartilage proteoglycan monomer (Figure 8b). As no excluded material is seen, it may be concluded that hyaluronic acid is absent from this fraction. Analysis of the fractions (A1D1-A1D3) confirmed the absence of hyaluronic acid in the A1D1 fraction (Table 6) and indicated that it is predominately in A1D3. Analysis of A1D1, A1D2, and A1D3 by polyacrylamide gel electrophoresis in SDS (Figure 11) showed that the link proteins are predominantly in fraction A1D3, with a trace in A1D2. Quantitation by a radioimmunoassay procedure specific for link protein (Caterson et al., 1980) confirmed these findings and indicated that 95% of the link proteins remained in the A1D3 fraction (Table 6). Therefore, a proteoglycan aggregate preparation, A1, layered on a preformed "dissociative" CsCl density gradient and centrifuged in the Beckman Airfuge for 4 h satisfactorily separates proteoglycan monomer (A1D1) from hyaluronic acid and link proteins (A1D3).

Mouse Colony at UAB

Breeding

Four to ten breeding pairs, age 6 weeks, of each strain, STR/N and STR/1N, were sent from the NIH Genetics Unit and used to establish

Figure 11 Polyacrylamide gel electrophoresis in SDS of fractions from a preformed dissociative CsCl density gradient fractionation of BNC A1 in the Beckman Airfuge. Gradient fractions were dialyzed against deionized water, lyophilized, and redissolved in sample buffer for electrophoresis. Bands were stained with Coomassie blue R. a and f, BNC link proteins; b, c, and d, A1D1, A1D2, and A1D3, respectively; e, BNC A1. T = top of gel.


T
a
b
c
d
e
f

a colony of mice at UAB animal facilities. Several difficulties were encountered. Not uncommonly, STR/N mice, at approximately 15 days of age (pre-weaning), were found dead in their cages. Not all of a litter would die, although sometimes as many as 50% would expire. Although never documented, it was suggested that these deaths were due to mouse hepatitis virus (Personal Communication, Dr. Nancy Cox, UAB Department of Comparative Medicine). Otherwise, the STR/N strain bred well. Unfortunately, the STR/1N strain does not breed as well and the number of 1N animals was usually limited. Poor breeding required that breeders be re-ordered several times. Although the cause of this infertility is not known, it may be related to the obstructive genitourinary disease in the STR/1N males (Sokoloff & Barile, 1962). Furthermore, between the ages of 6 and 12 months, 50% of STR/1N males die from this obstructive uropathy. Thus, poor breeding and high mortality in the STR/1N greatly limits the number of animals of all ages.

Osteoarthritis

The development of osteoarthritis in the mice bred at UAB was documented by histology and scanning electron microscopy.

Histology

Animals of both strains at several ages were sacrificed for histological verification of the osteoarthritic lesions. A comparison of 6-month-old 1N and N animals (Figure 12) illustrates the typical lesion as described by Sokoloff (1956). This lesion was seen most often in 1N mice as compared to N mice (Table 8).

Figure 12 Histological sections of hind knee joints from STR/N and STR/1N mice showing osteoarthritic lesion. Top panel, STR/N, 6 mo.; Bottom panel, STR/1N, 6 mo. Hematoxylin and eosin, magnification x 16.

TABLE 8

OCCURRENCE OF OSTEOARTHRITIC LESION IN UAB MOUSE COLONY

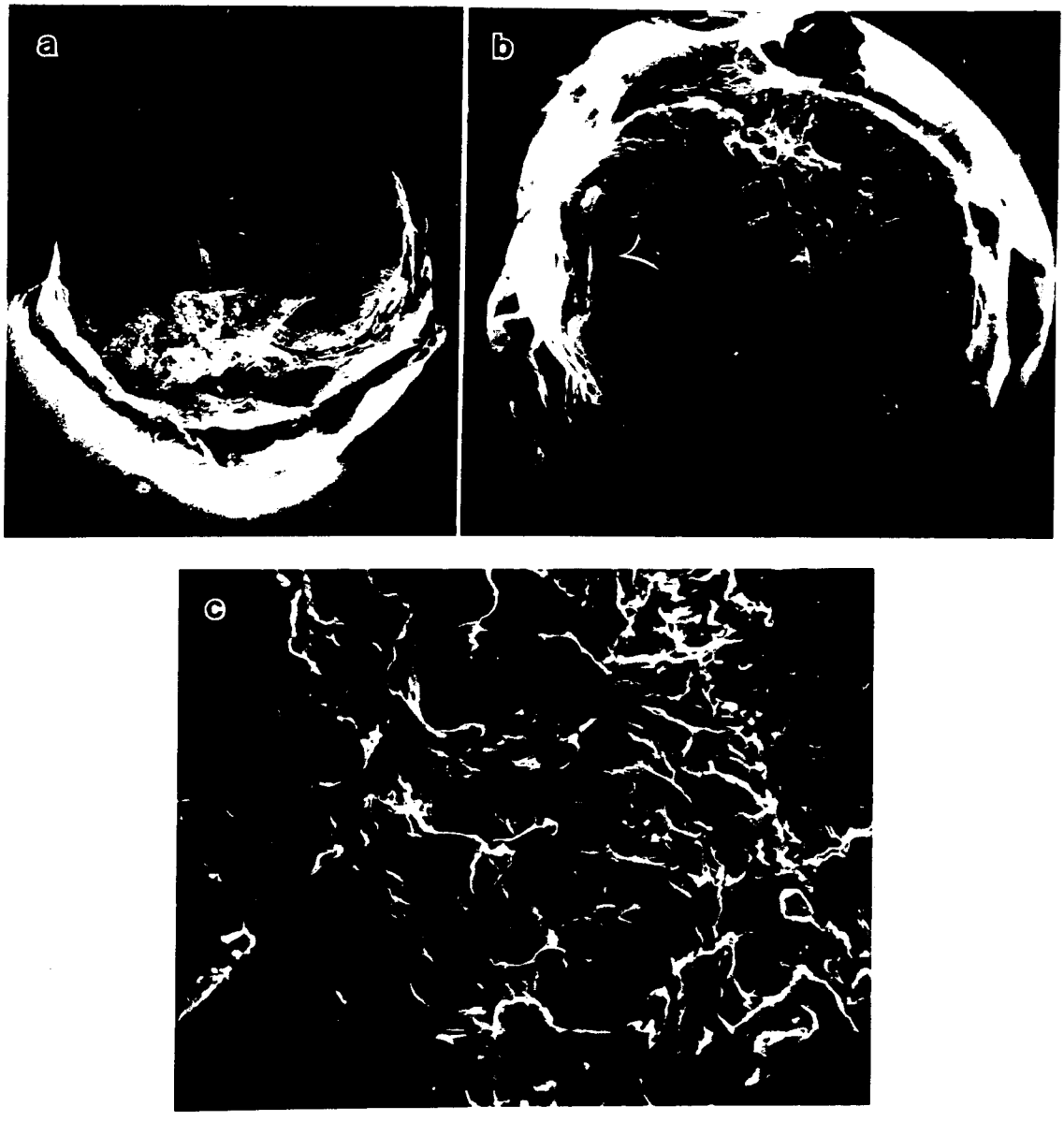
AGE (months)	Osteoarthritic Lesion* STR/N	STR/1N
1-3	0 (3)	0 (5), + (1)
4-6	0 (5)	+ (3)
7-12	0 (7)	+ (2)
> 12	0 (8) + (1)	+ (7)

* 0 = no histological evidence of OA
+ = at least 1+ OA (Sokoloff, 1956; see Figure 7)

Scanning Electron Microscopy (SEM)

One young (2 months) and one old (6 months) animal from each strain were sacrificed for analysis by SEM. The tibial surface from the 2 mo. old STR/N mouse is shown (Figure 13a) as an example of normal-appearing articular cartilage. The lesion on the tibial surface from the 6 mo. old STR/1N mouse (Figure 13b) was compared with that described by Walton (1977c). The lesion is delineated by a deep cleft in the cartilage (indicated by arrow in Figure 13b). Within this cleft the cartilage is deeply fibrillated (Figure 13c). According to Walton (1977c, p. 213), "The developing lesion covered an area adjacent to the cruciate ligament and consisted of exposed calcified cartilage which was bounded by a band of fibrillated uncalcified cartilage." No lesions were seen on the femoral surfaces; this is consistent with Walton's study which showed that the femoral lesions appeared much later than the tibial lesions.

Figure 13 Scanning electron micrographs of mouse knee joint articulating surfaces. a) STR/N, 2 mo, tibial surface, magnification x26. b) STR/1N, 6 mo, tibial surface, arrow indicates crescent-shaped cleft; magnification x26. c) lesion on STR/1N condyle, magnification x1200.


a
b
c

In Vivo Incorporation of ^{35}S-Sulfate

A preliminary experiment was carried out to determine the optimum time period for incorporation of ^{35}S-sulfate into non-dialyzable material. Three Swiss mice, age 2 mo., were each injected with 0.5 mCi ^{35}S-sulfate and sacrificed 10, 18, and 26 h later. The articular cartilage from the two hind knee joints of each mouse was extracted with 4 M GuHCl (1.0 ml) for 25 h at 4°C in the presence of protease inhibitors and carrier BNC A1,2 proteoglycan (1 mg/ml). The extracts were dialyzed to remove free sulfate and the total non-dialyzable ^{35}S-sulfate counts determined. The residues were digested with papain and total non-dialyzable counts determined for them as well. The greatest incorporation of radioactivity in the tissue occurred at 18 h (Table 9). The percentage extracted (98%) was the same in all cases. Subsequently, the 18 h time period was used for all in vivo incorporation studies.

TABLE 9

IN VIVO INCORPORATION OF ^{35}S-SULFATE INTO MOUSE ARTICULAR CARTILAGE AS A FUNCTION OF TIME

	10 h	18 h	26 h
		cpm	
4 M Extract	18×10^3	29×10^3	23×10^3
Residue	1.1×10^3	1.8×10^3	1.6×10^3

Young mice (< 4 mo.) incorporated the most ^{35}S-sulfate under these conditions. The low incorporation seen in older animals was consistently a problem and greatly limited the variety of analyses that could be done. Administration of the radiolabel in three separate doses over the 18 h period did not increase the level of incorporation.

Extraction of ^{35}S-Proteoglycans from Mouse Articular Cartilage

Since a criticism of earlier studies of osteoarthritis was the possibility of degradation during extraction of the proteoglycans, conditions of extraction were carefully evaluated. Articular cartilage of Swiss mice was labelled in vivo. The ^{35}S-proteoglycans were extracted from the cartilage either by direct extraction with 4M GuHCl or sequentially by extraction with 0.4 M GuHCl followed by 4M GuHCl. In either case, protease inhibitors were always present in the extraction mixture. A low extraction temperature was also important in minimizing degradation. Salt-ice baths were used to keep temperatures between 0°C and -10°C. Only with 4 M GuHCl extractions could the lowest temperatures be used since the high salt concentration prevented freezing.

In addition to the use of protease inhibitors and lowered temperatures, the time period of extraction was optimized for extraction of a representative population of proteoglycans while minimizing degradation. Aliquots of a 4 M GuHCl extract were taken at 5, 20, and 25 h and the amount of ^{35}S-sulfate determined (Table 10). Although most

TABLE 10

EXTRACTION OF ^{35}S-SULFATE FROM MOUSE ARTICULAR CARTILAGE AS A FUNCTION OF TIME

Incorporation Time in vivo (hrs)	Extraction Time (hrs) 5	10	25
10	20,700*	19,899	29,302
18	30,000	35,739	36,946
26	24,700	26,532	30,282

* 10 µl aliquots taken for counting; cpm adjusted for volume of extract. Total extraction volume equals 1 ml.

other studies Have used an extraction time of 48 h, this is probably unnecessarily long for our experiments since two-thirds or more of the radioactivity is solubilized between 5-10 h. This shorter time period (5-10 h) was chosen for all further extractions, thereby minimizing the time for possible degradation as well as accelerating the whole extraction and isolation process.

During a sequential extraction, the 0.4 M GuHCl extraction was allowed to proceed for 5 h, followed by a 5 h 4 M GuHCl extraction. For comparison, two sequential 5 h extractions with 4 M GuHCl were done. All extracts were dialyzed and the residues digested with papain. The total non-dialyzable ^{35}S-sulfate was determined for each fraction and the percentage of total counts solubilized was calculated (Table 11). Typically, whether sequential 0.4 M/4.0 M (0.4 M/S, 4.0 M/S) extraction was used or direct 4.0 M extraction,

TABLE 11

DISTRIBUTION OF ^{35}S-LABELLED MACROMOLECULES IN VARIOUS EXTRACTION FRACTIONS

Extraction Fraction	Non-Dialyzable ^{35}S-sulfate (% Total)
0.4 M	19
4.0 M	44
Residue	37
4.0 M	53
4.0 M	10
Residue	37

60-65% of the ^{35}S-sulfate macromolecules could be extracted from the cartilage.

To determine if the amount of low salt extractable proteoglycan increased in mouse osteoarthritic cartilage, the 0.4 M/4 M sequential extraction protocol was applied to articular cartilage from normal and osteoarthritic mice at ages ranging from 2 mos to 9 mos. These results are shown in Table 12. No progressive change in extractability with increasing age was seen in either normal or osteoarthritic animals (not shown), so results from all ages were averaged. No increase was observed in low salt extractable ^{35}S-sulfate from osteoarthritic as compared to normal mice, 19% vs. 20%, respectively. The data do suggest an increase in <u>total</u> extractable ^{35}S-sulfate in the osteoarthritic animals as compared to the normal, 73% vs. 64% and 59%, respectively. Results presented later will reveal that this increase in

TABLE 12

EXTRACTION OF ^{35}S-PROTEOGLYCANS FROM NORMAL AND OSTEOARTHRITIC MOUSE ARTICULAR CARTILAGE

		Extraction Fraction	Non-Dialyzable ^{35}S-Sulfate (% total)
Normal*	n = 6	4.0 M	64 ± 6+
		Residue	36 ± 6+
	n = 3	0.4	19 ± 4
		4.0	40 ± 3
		Residue	41 ± 6
Osteoarthritic	n = 6	0.4	20 ± 7
		4.0	53 ± 7+
		Residue	26 ± 8+

* Normal includes data from STR/N and Swiss mice.

\+ $p < 0.05$, Student's t-test for unpaired data.

extractability is not due to increased degradation of the ^{35}S-proteoglycan in the tissue resulting in increased solubilization.

Isolation of the A1 Fractions from Cartilage Extracts Using Preformed Gradients in the Airfuge

Since a change in the composition of proteoglycans from osteoarthritic animals might be reflected in their distribution in CsCl density gradients, it was important to examine the fractionation of proteoglycan in extracts using the Airfuge. Fractionation of BNC extracts had been studied extensively and extracts of other cartilages, particularly articular cartilages, were examined as well.

Cartilage extracts (4 M/S) were centrifuged under associative conditions on preformed gradients in the Airfuge and analyzed for distribution of uronic acid or ^{35}S-sulfate in fractions A1, A2, and A3 (Table 13). Mouse knee articular cartilage was never available in sufficient quantity to measure uronic acid. Although the data suggest a slight increase in the relative amount of ^{35}S-sulfate found in the osteoarthritic A1's as compared with the normal A1's, these numbers are not significantly different ($p > 0.10$). Only the results for 4 M/S GuHCl extracts are shown, but when analyzed, no different

TABLE 13

FRACTIONATION OF CARTILAGE EXTRACTS IN PREFORMED CsCl DENSITY GRADIENTS UNDER ASSOCIATIVE CONDITIONS IN THE AIRFUGE

Cartilage Extract	Distribution of Uronate or ^{35}S-Sulfate		
	A1	A2	A3
	%		
Bovine nasal septum	95	3	2
Sheep nasal septum	78	13	9
Rat chondrosarcoma	77	4	19
Sheep articular	72	18	10
Human knee			
normal	69	14	17
osteoarthritic	78	5	17
Mouse knee			
normal (n = 7)	62±15*	16±8	22±10
osteoarthritic (n = 6)	70±14	8±4	21±10

± = Standard deviation

* All mouse results are calculated from ^{35}S-sulfate cpm.

distribution was observed for associative (i.e., 0.4 M GuHCl) extracts than for 4 M extracts.

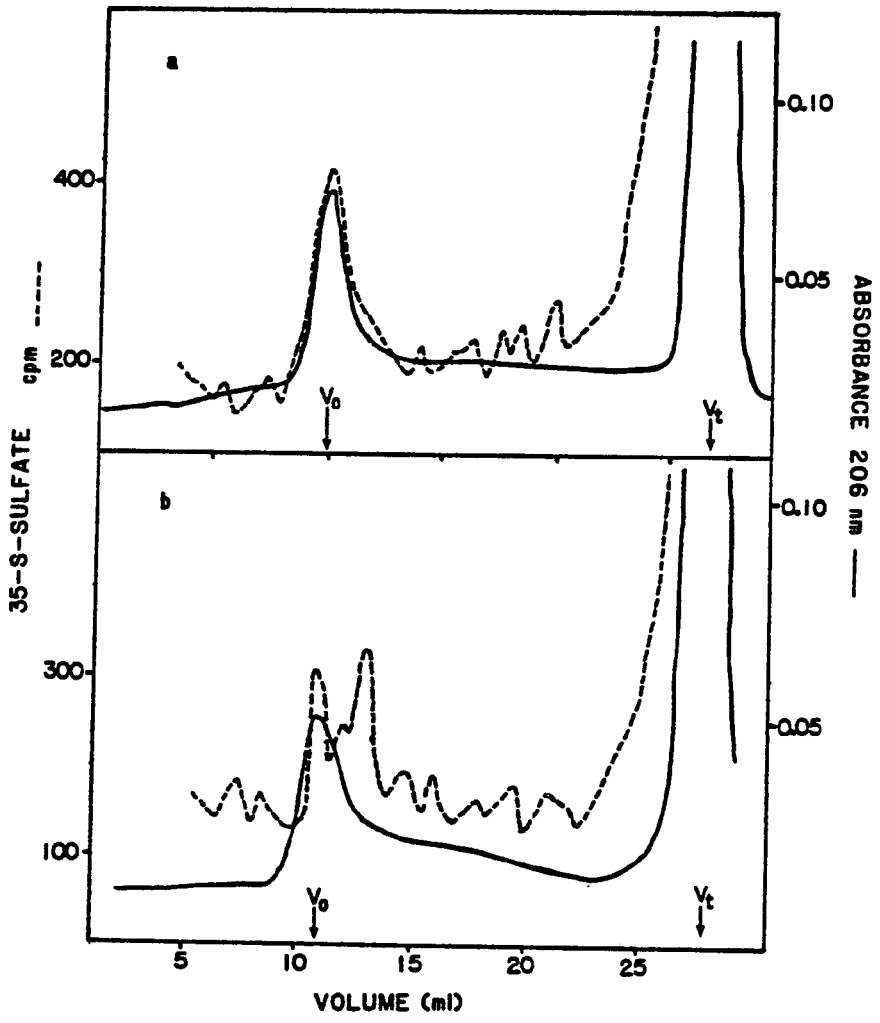
Analysis of ^{35}S-Proteoglycans from Normal and Osteoarthritic Mouse Articular Cartilage

Aggregation of ^{35}S-Proteoglycans

Associatively extractable PG. In one experiment only was the amount of ^{35}S-PG extracted by 0.4 M GuHCl enough to obtain a profile on Sepharose CL-2B (Figure 14). The profile for the STR/1N (Figure 14a) suggests that the majority of these associatively extractable PG are capable of aggregating. Although there is an excluded peak of ^{35}S-PG from the STR/N mice (Figure 14b), there is more included material in this profile than in the STR/1N profile. However, this particular experiment was done in the absence of carrier PG and it is therefore possible that degradation occurred during the extraction and isolation procedure. The 4 M/S ^{35}S-PG from this same experiment appears to be capable of aggregating (see "Analysis of Endogenous Proteoglycans"). It may be that the 0.4 M extraction brings together the normal endogenous proteases as well as extractable aggregates, allowing degradation to occur. Since no increase in associatively extractable ^{35}S-PG was seen in OA animals as compared to normals further studies focused on analysis of the 4 M GuHCl extracts.

Dissociatively extractable PG The ^{35}S-PG extracted by 4 M GuHCl, either directly or sequentially, was reassociated by dialysis to 0.4 M GuHCl and the Airfuge A1 fractions isolated. Chromatography on

Figure 14 Sepharose CL-2B chromatography of associatively extracted $^{35}SO_4$-proteoglycan from STR/1N and STR/N mouse articular cartilage. a. STR/1N; b. STR/N.


a
b
400
200
300
100
0.10
0.05
0.10
0.05
V_o
V_t
V_o
V_t
5
10
15
20
25
VOLUME (ml)
35-S-SULFATE cpm -----
ABSORBANCE 206 nm ——

Sepharose CL-2B was used to judge the ability of the ^{35}S-PG from osteoarthritic mice to aggregate. Elution profiles of the A1 fractions from osteoarthritic (STR/1N) mice, ages 4, 7, and 8 mo. (Figure 15), and STR/N and STR/1N mice, 2 and 6 mo. (Figure 16), are shown. In order to get some idea of the proportion of aggregate and monomer in these preparations, the profile for the 7-month-old STR/1N mouse was divided into two peaks, excluded and included; peaks were cut-out and weighed. The profile for the 7-month-old STR/N mouse (not shown) was treated similarly, as were the carrier profiles (A_{206}). The proportions of aggregate:monomer determined in this way were: STR/N, 65:35 (^{35}S), 60:40 (A_{206}); STR/1N, 75:25 (^{35}S), 70:30 (A_{206}). These profiles (Figures 15 and 16) show that the majority of PG monomers in these 4 M/S extracts are capable of aggregating. (One profile, Figure 16 female, 6 mo., appears to have a lower proportion of excluded material than the other profiles; the carrier PG reflects this as well indicating that there may have been degradation during the extraction and isolation of this sample.)

Gel Chromatography of Proteoglycan Monomers

Chromatography on Sepharose CL-2B was used to compare the hydrodynamic size of proteoglycan monomers from normal and osteoarthritic mouse articular cartilage. As a basis for comparison the weight average molecular weight ($\bar{M}w$) of BNC monomer, of known K_{AV} on Sepharose CL-2B, was measured in the analytical ultracentrifuge.

Figure 15 Sepharose CL-2B chromatography of $^{35}SO_4$-proteoglycans from osteoarthritic mouse articular cartilage, ages 4, 7, and 8 months. The 4 M/S extraction was done in the presence of carrier BNC A1; absorbance at 206 nm indicates the elution profile of the carrier PG. a. 4 months; b. 8 months; c. 7 months.

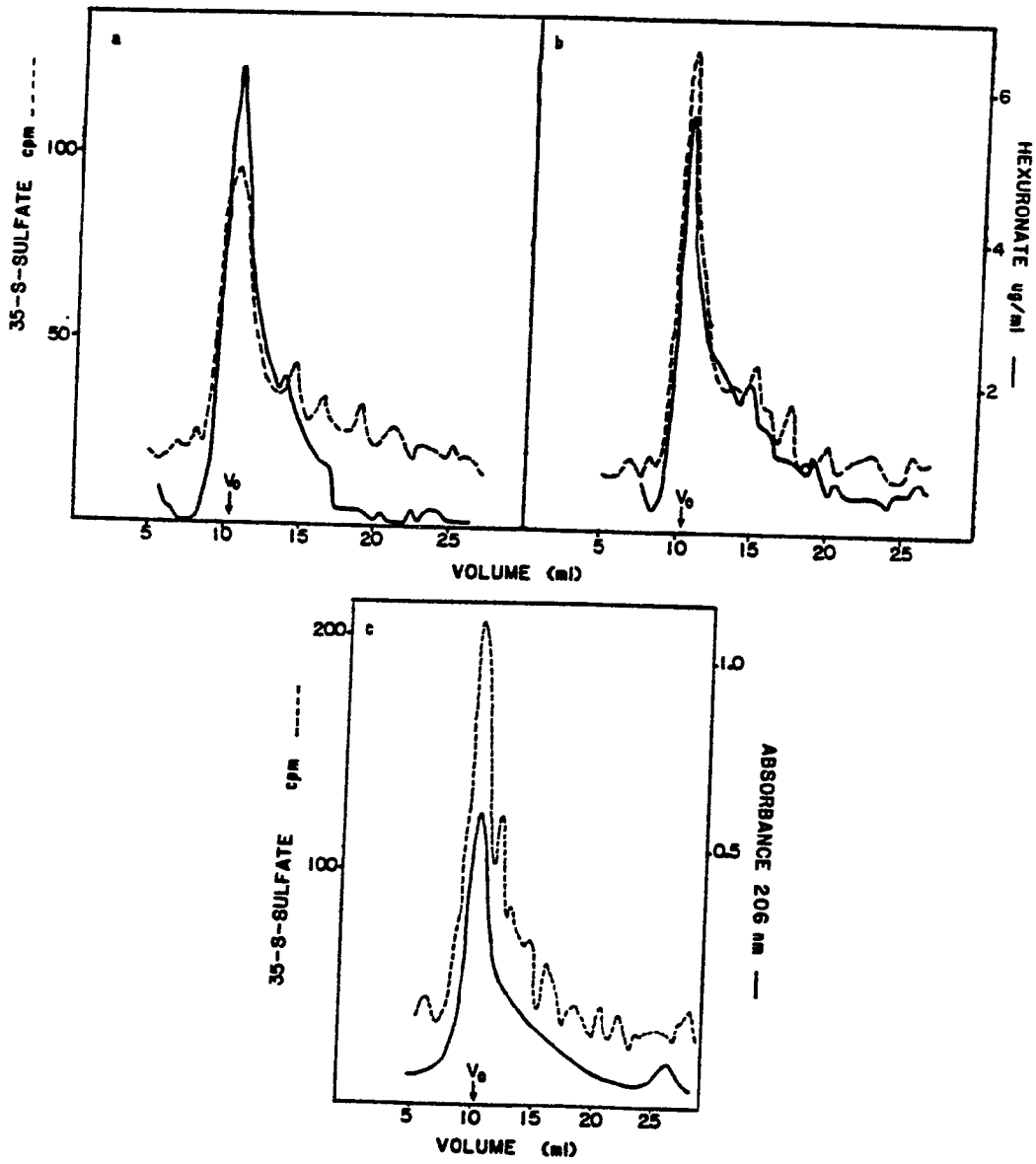
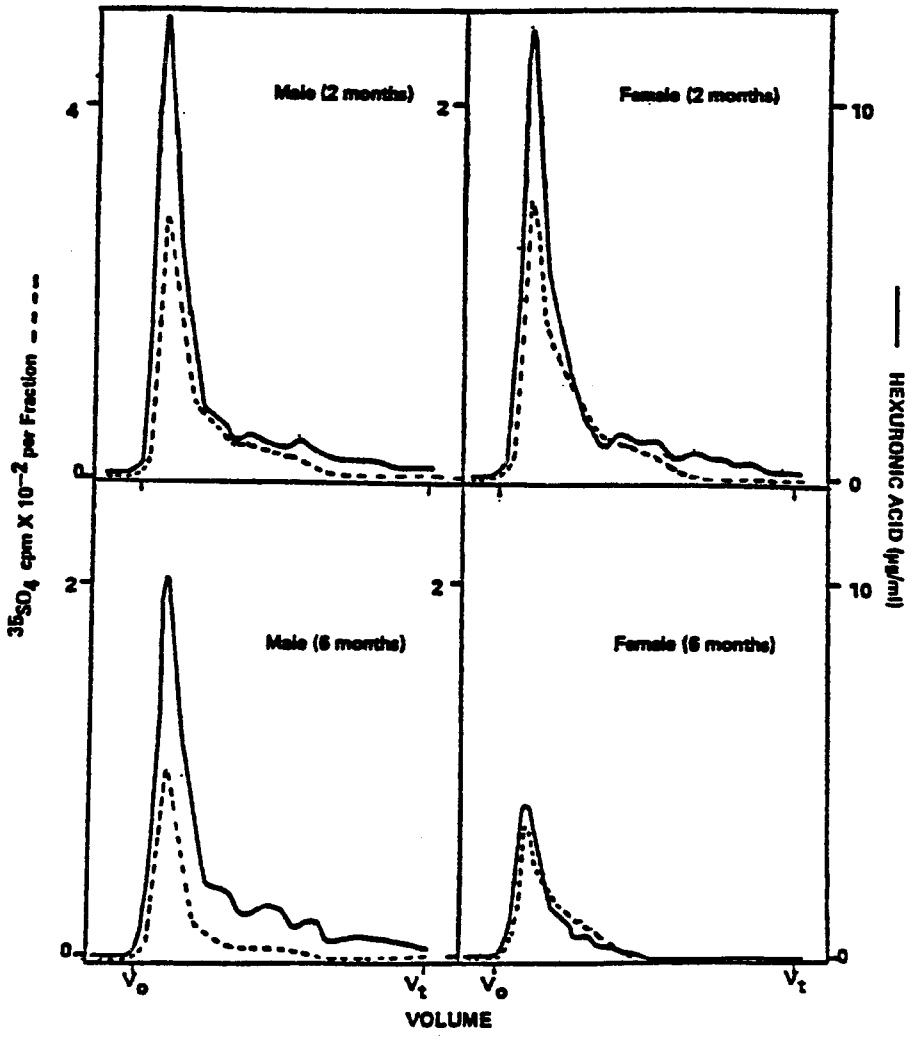

a
b
c
35-S-SULFATE cpm
100
50
200
100
HEXURONATE µg/ml
6
4
2
ABSORBANCE 206 nm
1.0
0.5
V_0
5
10
15
20
25
VOLUME (ml)

Figure 16 Sepharose CL-2B chromatography of $^{35}SO_4$-proteoglycans from osteoarthritic mouse articular cartilage, ages 2 and 6 months. The 4 M/S extraction was done in the presence of carrier BNC A1. Absorbance at 206 nm indicates the elution profile of the carrier PG.


Male (2 months)
Female (2 months)
Male (6 months)
Female (6 months)
$^{35}SO_4$ cpm X 10^{-2} per Fraction - - - -
HEXURONIC ACID (μg/ml)
V_o
V_t
VOLUME

Molecular Weight of BNC A1D1

Velocity runs at two different concentrations of proteoglycan revealed the concentration dependence of the sedimentation coefficient (Figure 17). The sedimentation coefficient extrapolated to infinite dilution, $S^{o}_{20,w}$, equals 25S, in good agreement with that reported by Hopwood and Robinson (1975), $S^{o}_{20},w = 26S$. Hopwood and Robinson (1975) also found that at proteoglycan concentrations of 0.2 mg/ml and below, the molecular weight as determined by equilibrium sedimentation (meniscus depletion method, Yphantis, 1964) did not vary significantly with concentration. Therefore similarly low concentrations were chosen for this study. The results of measuring fringe displacement at varying radial distance from the center of rotation are presented graphically in Figure 18. The molecular weight calculated from these data is 1.5×10^6. Again this is in reasonable agreement with Hopwood and Robinson (1977) who reported 1.2×10^6.

^{35}S-Proteoglycan Monomers from Normal and Osteoarthritic Mice

The size of ^{35}S-PG monomers from mouse articular cartilage was analyzed by their elution from Sepharose CL-2B. The articular cartilage was labelled *in vivo*, extracted with 4 M GuHCl, and the monomer fraction (D1) isolated in the Airfuge on dissociative gradients. It is quicker to obtain a D1 directly and, in general, more radioactivity could be recovered by direct dissociative isolation than by isolation of an A1D1. This increased recovery is probably due to two factors, fewer manipulations of the sample and inclusion of the total monomer population in the D1 rather than just the subpopulation of the A1D1.

Figure 17 Concentration dependence of the sedimentation coefficient for BNC A1D1 in the analytical ultracentrifuge. The velocity was 56,000 RPM.

x---x---x, dry weight estimated by protein content.

·---·---·, dry weight estimated by uronic acid content.

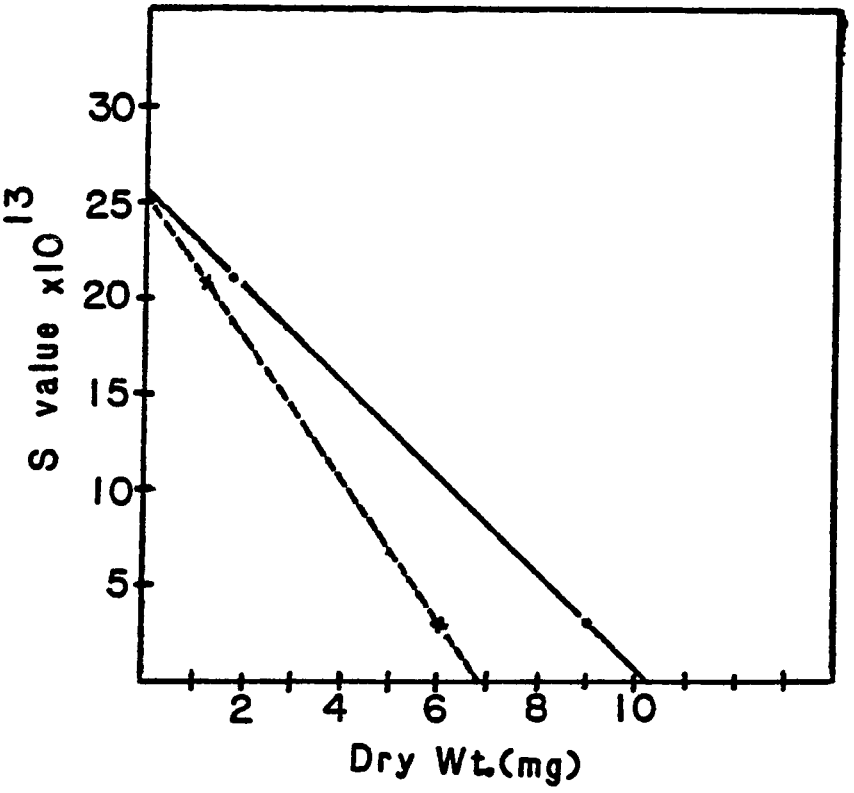
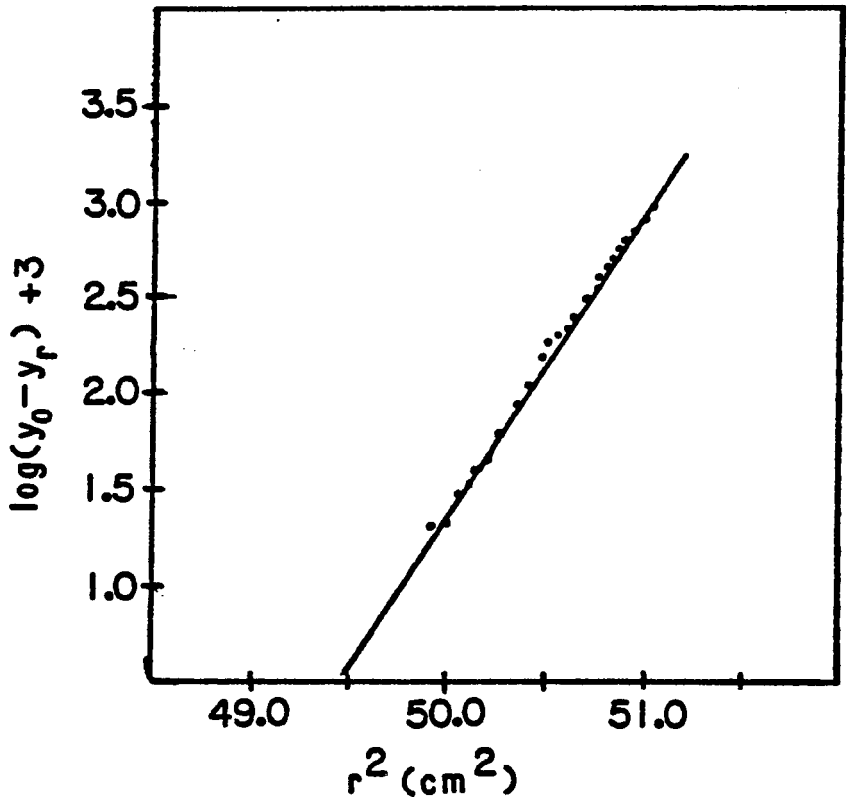

30
25
20
15
10
5
S value x10 13
2
4
6
8
10
Dry Wt.(mg)

Figure 18 Determination of the molecular weight of BNC A1D1 by sedimentation equilibrium in the analytical ultracentrifuge. The velocity was 4,800 RPM. Proteoglycan was in 0.1 M NaCl - 0.12 mM $NaHCo_3$ at a concentration of 0.2 mg/ml.


3.5
3.0
2.5
2.0
1.5
1.0
log(y0−yr) +3
49.0
50.0
51.0
r2 (cm2)

Carrier PG was present from the time of extraction in order to help minimize and monitor degradation and as an internal standard for the chromatography. Elution from the column was achieved with 4 M GuHCl in order to minimize non-specific binding of ^{35}S-PG molecules to each other or to the Sepharose. Monomers from Swiss mice, STR/N and STR/1N, were isolated and compared. Elution profiles were sometimes trimodal (Figure 19a). This could be due to the existence of three different populations of monomers (Hopwood & Robinson, 1975). Elution profiles of STR/N and STR/1N (Figure 19b and c) revealed intact ^{35}S-monomers and no difference in their K_{AV} (0.47 and 0.46, respectively). The data from several different experiments are summarized in Table 14 (the ^{125}I data will be discussed in the next section). Although too few determinations are available for a statistical analysis, no large differences are apparent. Monomers from STR/1N mice at 4 mo. and 5 mo. had K_{AV}'s (0.46 and 0.47, respectively) not unlike monomers from STR/N mice of the same ages (K_{AV}'s = 0.47 and 0.48, respectively). Furthermore, these K_{AV}'s are similar to the K_{AV} of the monomers from Swiss mice (Figure 19a, peak b). Obviously, mouse articular cartilage PG monomers are smaller than those from BNC (K_{AV} = 0.28). Other monomer preparations, e.g., RC and fBAC, were used as well to give some indication of the reproducibility of the column chromatography.

Analysis of Endogenous Proteoglycans

In vivo labelling with ^{35}S-sulfate was useful in studying the PG being synthesized by the chondrocytes during the period of

Figure 19 Sepharose CL-2B chromatography of $^{35}SO_4$-labelled mouse articular cartilage proteoglycan monomers. A. Normal Swiss mouse, A1D1; a, b and c indicate K_{av}'s of 0.28, 0.46 and 0.66, respectively. The absorbance at 206 nm indicates the elution profile of carrier bovine nasal cartilage A1D1 which has a K_{av} of 0.22. B. STR/N, D1, K_{av} = 0.48. C. STR/1N, D1, K_{av} = 0.47.

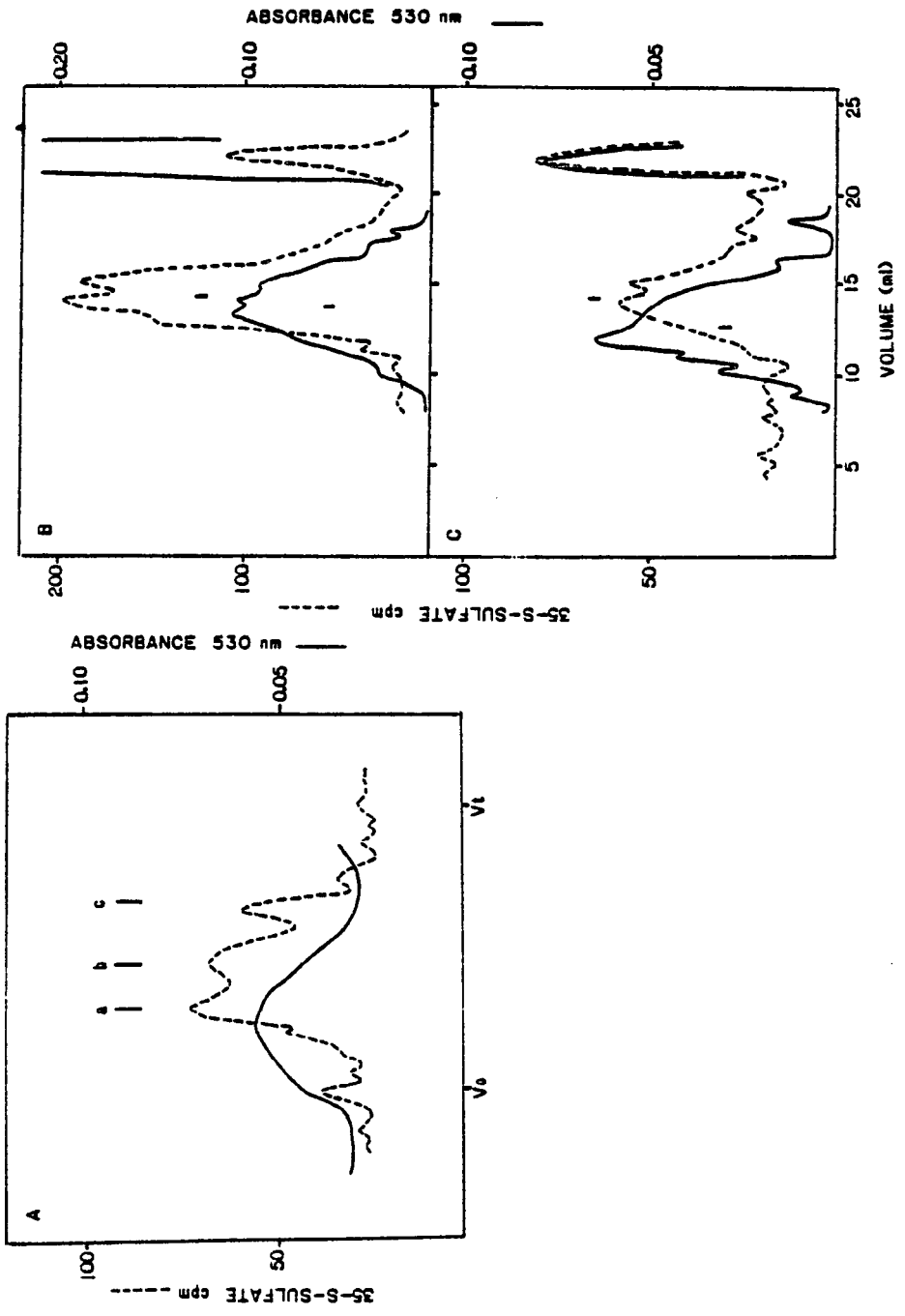

ABSORBANCE 530 nm
0.20
0.10
0.10
0.05
B
C
200
100
100
50
35-S-SULFATE cpm
5
10
15
20
25
VOLUME (ml)
ABSORBANCE 530 nm
0.10
0.05
a
b
c
Vt
Vo
A
100
50
35-S-SULFATE cpm

TABLE 14

SIZE OF PROTEOGLYCAN MONOMERS FROM STR/1N AND STR/N MOUSE ARTICULAR CARTILAGE

Strain	Age[1]	Mouse Monomer	K_{AV}[2]
STR/1N	4 mo.	^{35}S-D1	0.46 (0.39)[3]
	5 mo.		0.47 (0.43)
	--	^{125}I-A1D1	0.47 (0.34)
	4 mo.		0.45 (0.34)*
	6 mo.	^{125}I-A1	0.49
STR/N	4 mo.	^{35}S-D1	0.47 (0.34)
	5 mo.		0.48 (0.37)
	3 mo.	^{125}I-D1	0.41 (0.28)**

[1], Articular cartilage at least three mice from each age was pooled; [2], K_{AV} from radioactivity profile; [3], ( ), K_{AV} of carrier monomer from uronic acid profile; * fetal bovine articular cartilage monomer; ** bovine nasal cartilage monomer, all others rat chondrosarcoma monomer.

incorporation. But link protein(s) was not labelled by this method nor was any PG that had been synthesized prior to the in vivo labelling period (endogenous PG). To characterize the link proteins and the endogenous PG, articular cartilage was extracted in the absence of carrier PG, fractionated in the Airfuge either associatively or dissociatively, and the appropriate fractions iodinated (see "Methods"). In one experiment, the proteoglycans were labelled in vivo with $^{35}SO_4$. Thus, both the newly synthesized and endogenous PG in the same preparation could be compared.

The ^{125}I-A1 profile on Sepharose CL-2B (Figure 20b, d) revealed that the endogenous PG from both N and 1N animals is capable of aggregation. The $^{35}SO_4$ profile (Figure 20a, c) was similar to the ^{125}I profile and shows that degradation during extraction and isolation did not occur. The ^{125}I profile does suggest an increase in slightly included material. This included ^{125}I-PG may have resulted from degradation during the iodination procedure or may truly reflect the proportion of nonaggregated PG in the endogenous PG population.

Chromatography of an ^{125}I-D1 on Sepharose CL-2B eluted with 4 M GuHCl (Figure 21a) showed that the articular cartilage monomer (K_{AV} = 0.46) from a Swiss mouse is smaller than monomer from BNC (K_{AV} = 0.28) and also smaller than monomer from sheep articular cartilage (K_{AV} = 0.35). The sizes of the monomers from normal and osteoarthritic mice were compared in this way and the results are summarized in Table 14. Carrier PG monomer was included in each chromatographic run as an internal standard for comparison of different runs. The K_{AV} was 0.41 (n = 1) for monomers from STR/N and 0.46 (n = 2) for STR/1N). More

Figure 20 Sepharose CL-2B chromatography of $^{35}SO_4$- and ^{125}I-labeled proteoglycan aggregate from STR/N and STR/1N articular cartilage. Proteoglycan was labeled *in vivo* with $^{35}SO_4$, extracted and isolated in the absence of carrier PG, and the Airfuge A1 radiolabeled with ^{125}I. Top panels are STR/N, 9 mo., bottom panels are STR/1N, 9 mo.; left panels are $^{35}SO_4$-labeled PG, right panels are ^{125}I-labeled PG. Absorbance 206 nm is profile of carrier RC A1 added at time of chromatography.

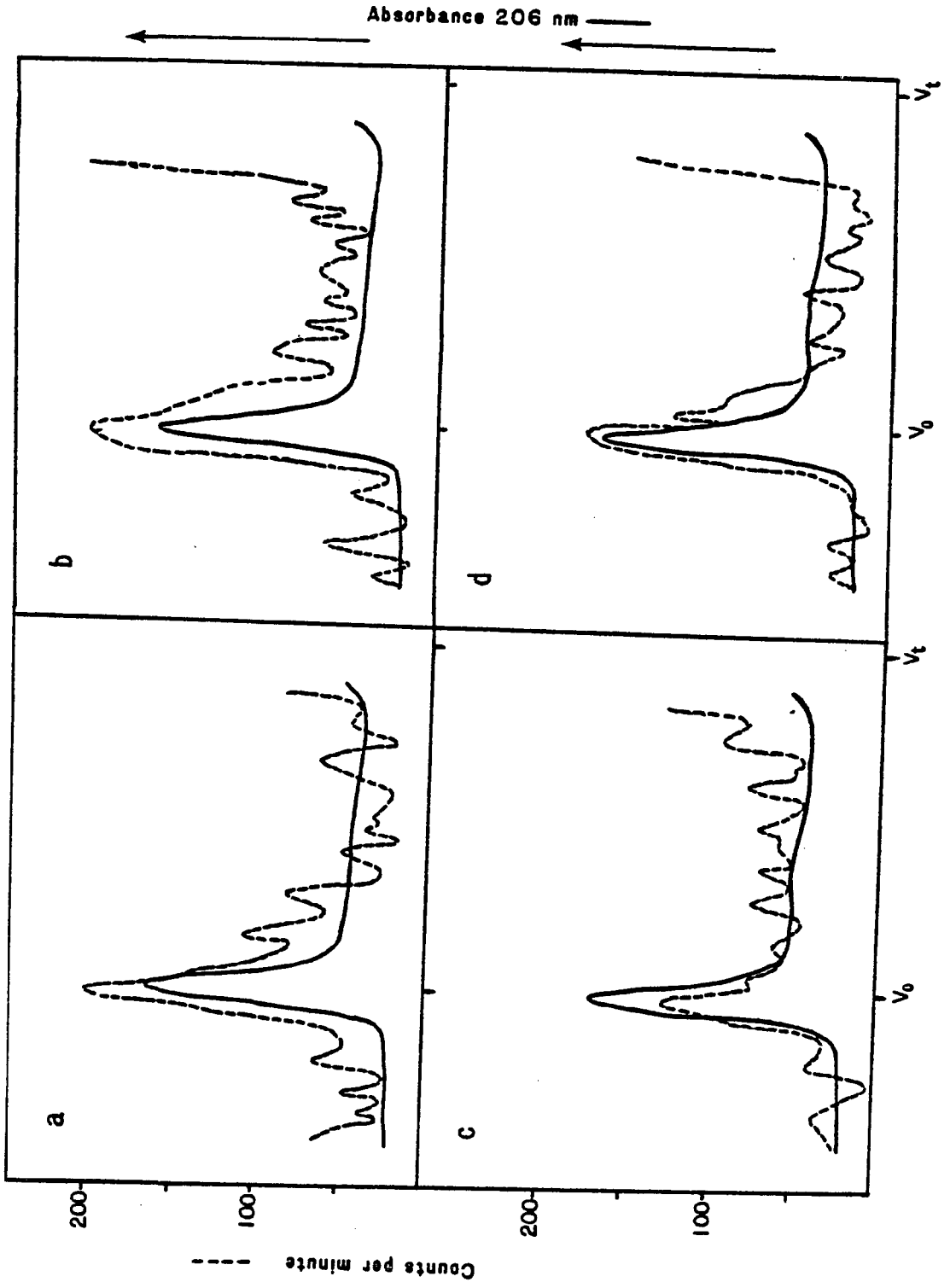
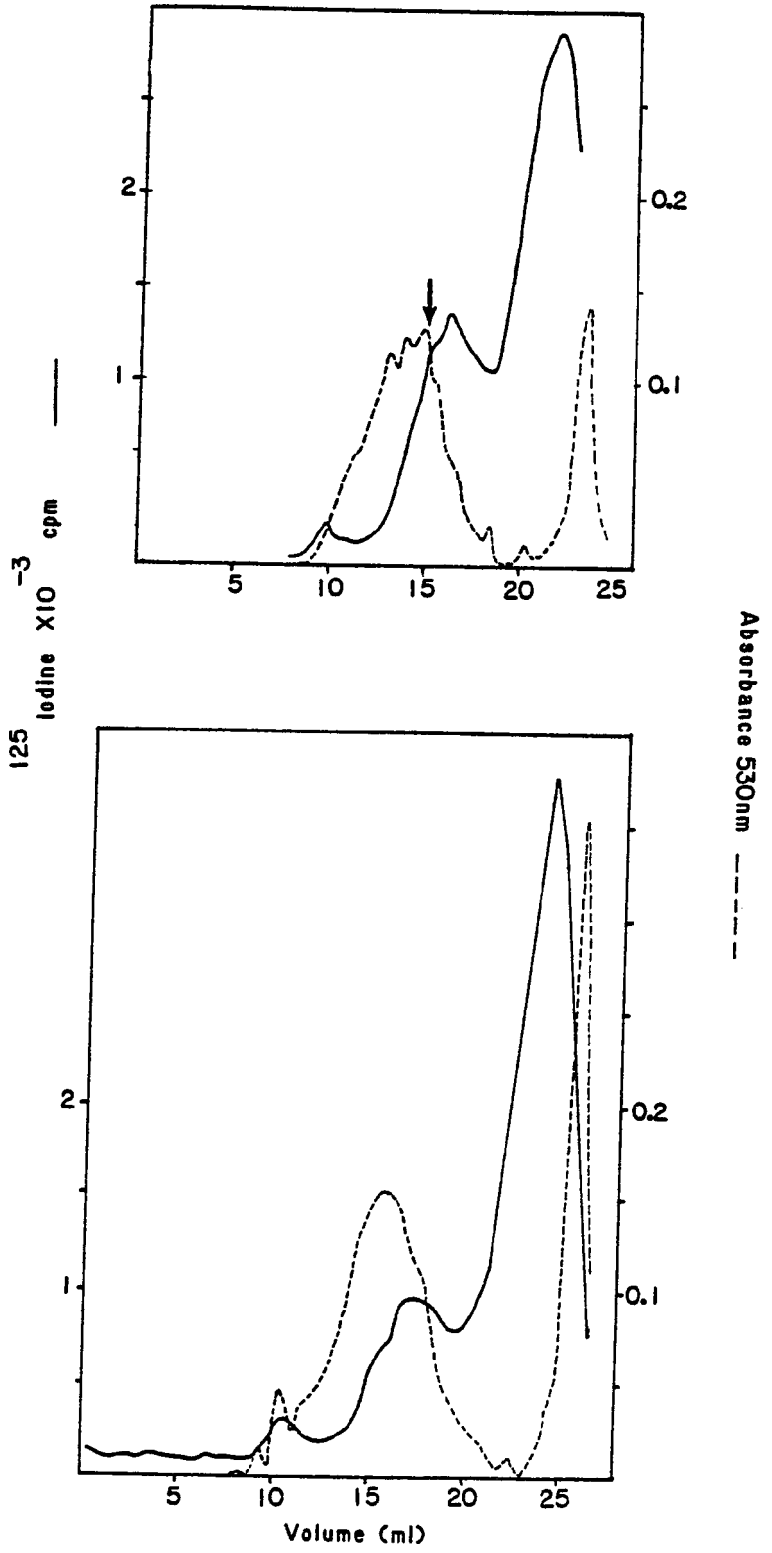

Absorbance 206 nm
a
b
c
d
V_o
V_t
V_o
V_t
200
100
200
100
Counts per minute

Figure 21 Sepharose CL-2B chromatography of ^{125}I-labeled proteoglycan monomer from STR/N and STR/1N articular cartilage. Direct D1 fractions were isolated in Airfuge in the absence of carrier PG and iodinated. Absorbance 530 nm is from the carbazole assay for uronic acid used to locate carrier RC monomer. Top panel, STR/N; bottom panel, STR/1N. ↓, elution position of monomer from sheep articular cartilage, $K_{AV} = 0.35$. STR/N, $K_{AV} = 0.41$; STR/1N, $K_{AV} = 0.45$.


125 Iodine X10 -3 cpm
Absorbance 530nm
Volume (ml)
2
1
0.2
0.1
5
10
15
20
25

data would have been desirable so that the K_{AV}'s could be compared statistically. Unfortunately, an excessive number of animals would be needed to obtain sufficient counts of ^{125}I in PG, under the mild radiolabelling conditions used.

Analysis of ^{35}S-Glycosaminoglycan Chains from Normal and Osteoarthritic Mouse Articular Cartilage

Gel Chromatography of Individual Glycosaminoglycan Chains

Diminished glycosaminoglycan chain size is one possible means of compromising proteoglycan function in osteoarthritic cartilage and had been reported by Bollet and Nance (1966). Whether this is true for the articular cartilage from osteoarthritic mice was examined.

Papain was used to degrade the proteoglycans in cartilage, releasing single chondroitin sulfate chains with only 4-5 amino acids still attached. After 24 h at 60-65°C, cartilage pieces were visibly changed with only a small amount of flocculant material remaining. All ^{35}S-labeled material was solubilized. The single GAG chains were analyzed by gel chromatography on Sepharose Cl-6B. Mouse articular cartilage from normal and osteoarthritic mice of matched ages was analyzed in this way. Typical profiles are shown in Figures 22 and 23. The ^{35}S-glycosaminoglycans from osteoarthritic mice had the same K_{AV} as those from normal mice: $K_{AV} = 0.61 \pm 0.04$ ($n = 5$), Swiss mouse; $K_{AV} = 0.62 \pm 0.03$ ($n = 4$), STR/N; $K_{AV} = 0.63 \pm 0.03$ ($n = 10$), STR/1N. No difference was seen when older and younger animals were compared (Figure 23). As expected for articular cartilage, these mouse

Figure 22 Sepharose CL-6B chromatography of ^{35}S-glycosaminoglycans from normal mouse articular cartilage. a) BNC GAG K_{AV} = 0.46; b) ^{35}S-GAG, K_{AV} = 0.62; c) free $^{35}SO_4$.

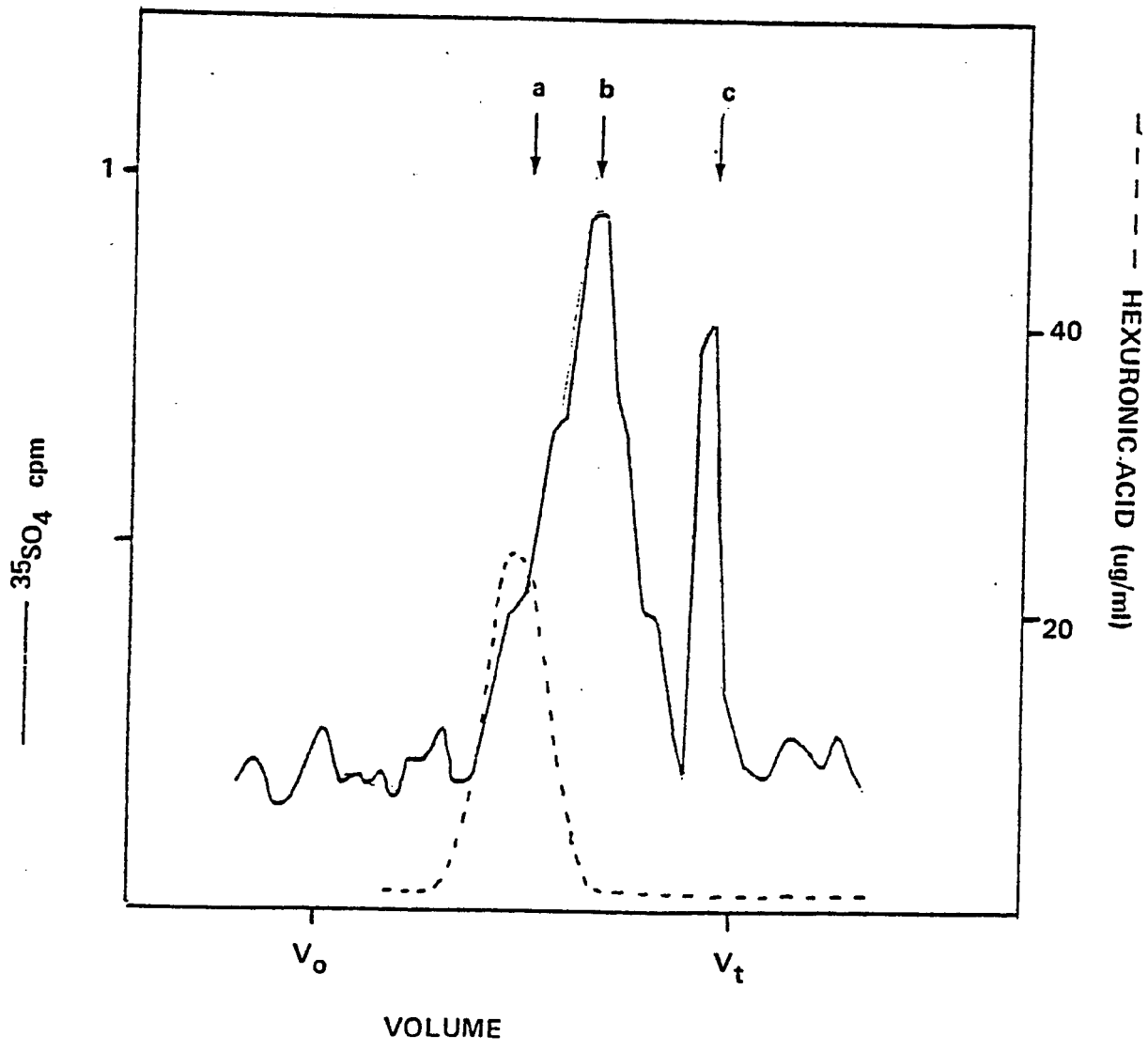
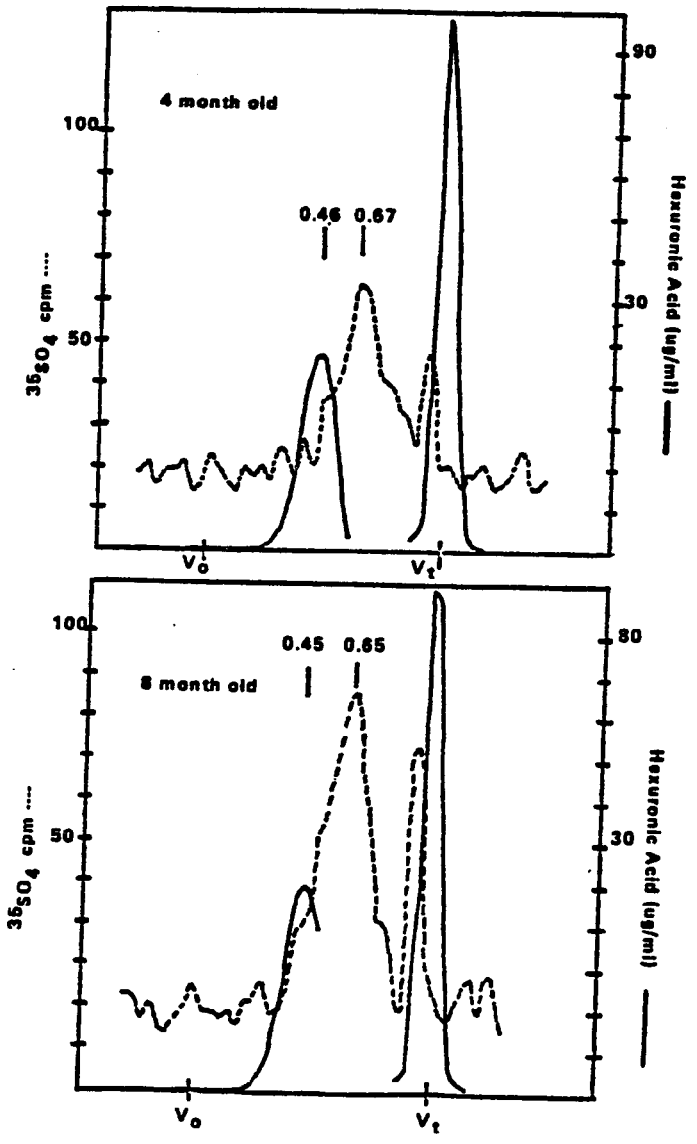

a
b
c
1
40
20
$^{35}SO_4$ cpm
HEXURONIC ACID (ug/ml)
V_o
V_t
VOLUME

Figure 23 Sepharose CL-6B chromatography of ^{35}S-glycosaminoglycans from osteoarthritic mouse articular cartilage.


4 month old
0.46
0.67
100
50
90
30
$^{35}SO_4$ cpm ....
Hexuronic Acid (ug/ml) ——
V_o
V_t
6 month old
0.45
0.65
100
50
80
30
$^{35}SO_4$ cpm ....
Hexuronic Acid (ug/ml) ——
V_o
V_t

glycosaminoglycans are smaller than those from bovine nasal cartilage, $K_{AV} = 0.46 \pm 0.03$ (n = 18).

The Sepharose CL-6B profiles were frequently trimodal for ^{35}S-sulfate. Whether or not this represents different size populations of chrondroitin sulfate chains and/or the presence of keratan sulfate chains is not known.

Chondroitinase Sensitivity of Glycosaminoglycans

The ^{35}S-glycosaminoglycans isolated from mouse articular cartilage were tested for their susceptibility to chondroitinases AC and ABC. The release of unsaturated disaccharides (ΔDi-0S, ΔDi-4S, and ΔDi-6S) from the carrier BNC GAG was followed by monitoring the absorbance at 232 nm (Figure 24). The digestion appeared complete after 3 h at room temperature but was usually allowed to continue for 5 h. The digestion mixture was applied directly to a Bio-gel P-4 column and the effluent analyzed for radioactivity (Figure 25). Typically, 85% of the radioactivity appeared in the sharp peak near V_T. The remainder was distributed between a V_0 peak and a broad included peak. The V_0 peak is material resistant to chondroitinase digestion, e.g., keratan sulfate. The included peak, sometimes more evident than in Figure 24, is linkage region oligosaccharide with 1-2 sulfated disaccharide units attached. No difference in the ^{35}S-sulfate distribution among these peaks was seen between normal and osteoarthritic age-matched mice (data not shown). This would suggest there is no difference in the amount of KS (V_0 peak) present in the osteoarthritic mouse

Figure 24 Digestion of carrier glycosaminoglycans by chondroitinase ABC as indicated by increased absorbance at 232 nm. a, b, c, d, are four different incubation mixtures in four different cuvettes.

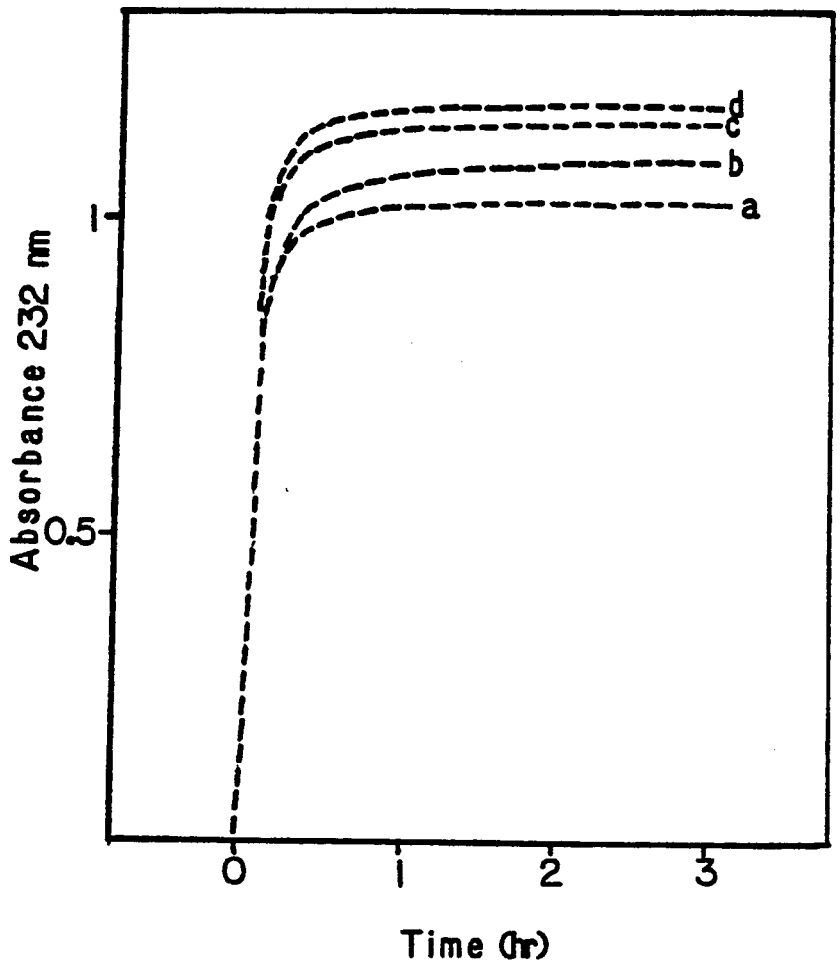
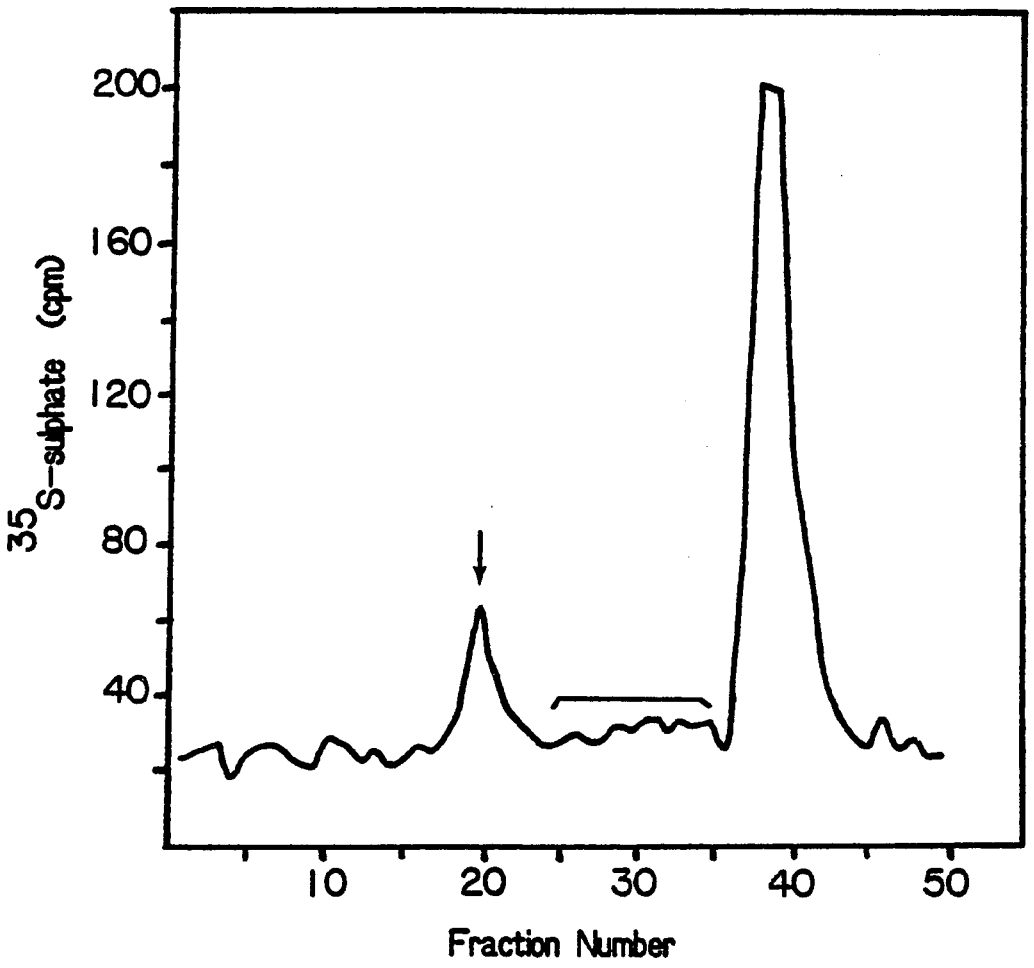

Absorbance 232 nm
1
0.5
0
1
2
3
Time (hr)
a
b
c
d

Figure 25 Chromatography on BioGel P-4 of a chondroitinase digest of mouse articular cartilage GAG. arrow indicates V_o; bracket indicates included region of gel.


200
160
120
80
40
35S-sulphate (cpm)
10
20
30
40
50
Fraction Number

cartilage when compared to normal mice of the same age. Also, dermatan sulfate does not appear to contribute significantly to the GAG population since addition of chondroitinase ABC to an AC digest did not result in further degradation (data not shown).

C-4-S and C-6-S Content of Glycosaminoglycans

Further analysis of the disaccharide peak (V_T, Bio-gel P-4) revealed the relative amounts of ΔDi-4S and ΔDi-6S. Non-sulfated disaccharide was not estimated since only ^{35}S-labelled material could be detected from the mouse cartilage. The isomeric disaccharides were separated either by thin-layer chromatography (TLC) or HPLC (see "Methods"). Analysis by TLC, although requiring less time than paper chromatography, was a lengthy and tedious process and did not always resolve the isomers sufficiently. Thus, TLC was abandoned for HPLC. Chromatography on a Waters carbohydrate column was monitored by absorbance at 232 nm. A typical elution profile (Figure 26) for disaccharides from carrier BNC GAG indicated two peaks of A_{232} at the positions for standards of ΔDi-4S and ΔD-6S. BNC GAG were 85% ± 4% (n = 7) ΔDi-4S. Radioactivity assays for ^{35}S-sulfate gave peaks coincident with the A_{232} peaks of ΔDi-4S and ΔDi-6S. The relative amounts of these two peaks were determined for age-matched normal and osteoarthritic mice (Table 15). The percent ΔDi-4S was highest in very young animals, ~ 90%, and remained > 50% at all ages. Although the percent ΔDi-4S for the youngest Swiss mice (80%) is less than for the 1N and N young animals, the Swiss mouse data were obtained by the TLC method which generally gave lower values than the HPLC method.

Figure 26 HPLC separation of 4-sulfated and 6-sulfated disaccharides from chondroitinase digestion of BNC GAG.

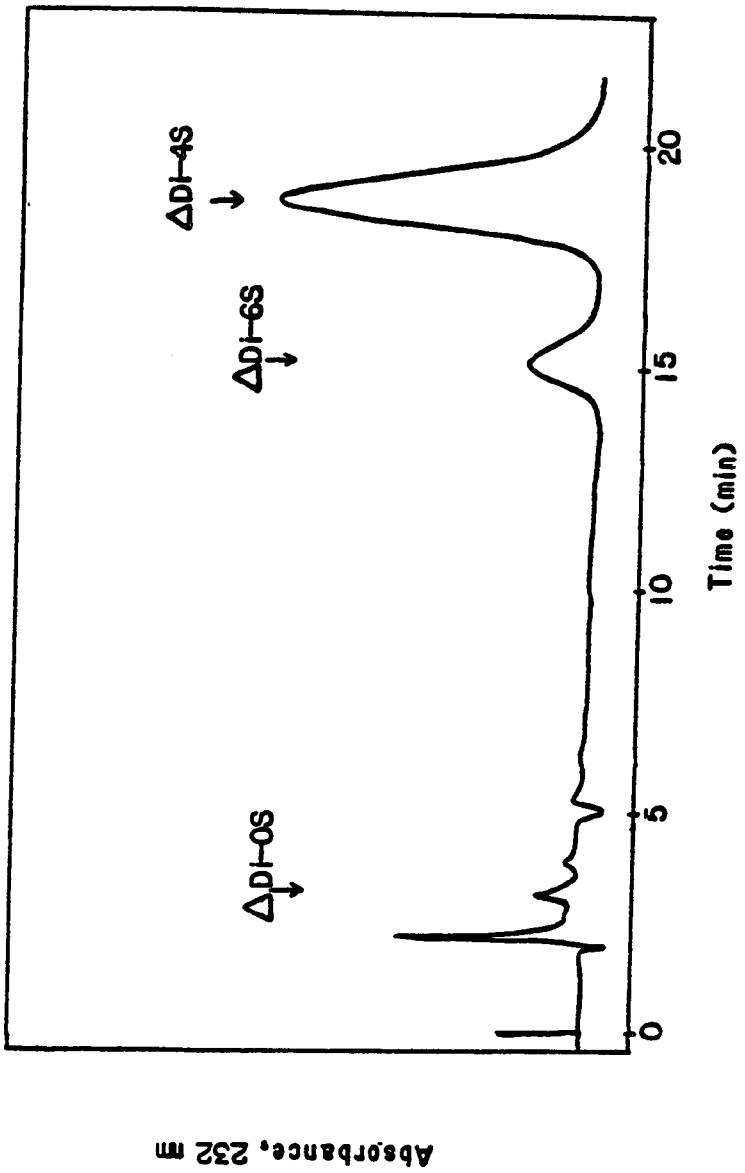

ΔDI-4S
ΔDI-6S
ΔDI-0S
0
5
10
15
20
Time (min)
Absorbance, 232 nm

TABLE 15

CHONDROITIN-4-SULFATE CONTENT OF ARTICULAR CARTILAGE FROM STR/N, STR/1N, AND SWISS (WM) MICE

MOUSE STRAIN[1]	PERCENT CHONDROITIN-4-SULFATE[2] 1 - 3 MO	4 - 6 MO	7 - 12 MO
1N	94 ± 2 (3)[3]	72 ± 7 (5)	76 ± 6 (6)
N	91 ± 8 (8)	--	81 ± 5 (5)
WM	80 ± 7 (3)	63 ± 4 (2)	77 ± 10 (5)

[1] BNC GAGS, 85% ± 4 (n = 7); rat chondrosarcoma GAGS, 98%.

[2] ΔDi-4-S + ΔDi-6-S = 100%.

[3] 1-month-old only.

Studies of articular cartilage in other animals have shown that the C-4-S content is > 50% in young animals and < 50% in older animals. These mouse strains do not appear to undergo this transition. The oldest mice, 7-12 mo., may have less C-4-S relative to C-6-S than the 1-3 mo. old mice, but the C-4-S isomer still predominates in the older animals. The osteoarthritic animals do not appear to have different amounts of C-4-S than the other two normal strains.

Level of Sulfation of Glycosaminoglycans

CPG-DEAE Chromatography of BNC GAG

Ion-exchange chromatography is often performed using DEAE-cellulose. A relatively new product, controlled-pore glass DEAE

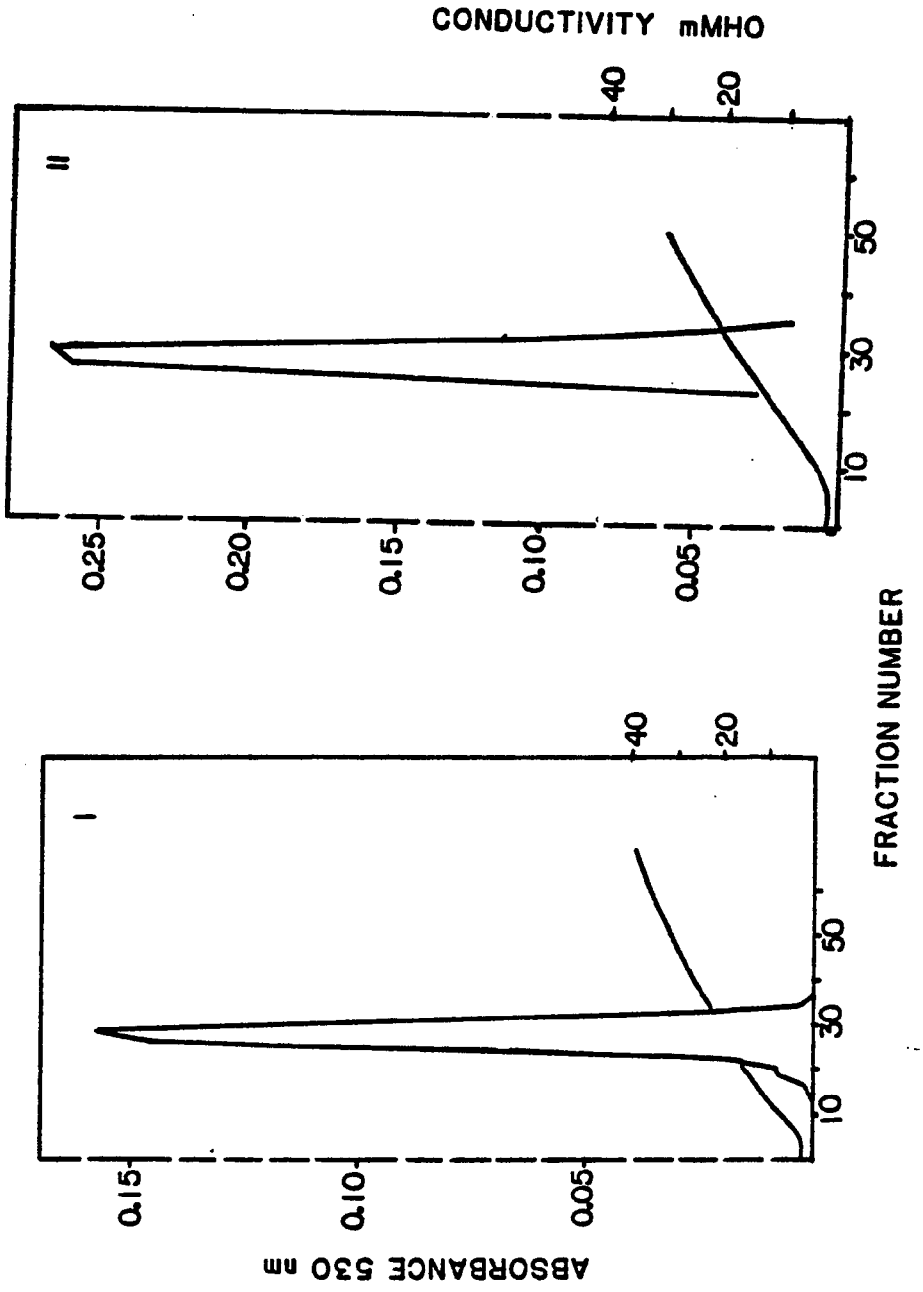
(CPG-DEAE), was tested as an alternative to the cellulose derivative since the glass beads can be used at high flow rates without shrinking or clogging. Repeated chromatography of a BNC glycosaminoglycans standard revealed the elution characteristics (Figure 27) of this preparation. Fractions across the elution peak were taken for sulfate and uronic acid analysis (see Analytic Methods). The starting material had a sulfate:uronic acid ratio of 0.92. Although there appeared to be a trend from lower to higher ratios as the elution position moved to higher ionic strength, the change from fraction to fraction was variable (Table 16). The earliest eluting material appeared to have a consistently high ratio. The variability of these results

TABLE 16

SULFATE TO URONATE RATIOS FOR BNC GLYCOSAMINOGLYCANS FRACTIONATED BY CPG-DEAE CHROMATOGRAPHY

Run No.	Fraction No.	Ratio
I	26	1.04
	27	0.82
	28	0.92
	29	1.03
	30	1.19
II	24	2.9
	25	0.73
	26	0.86
	27	0.47
	28	0.75
	29	0.74
	30	0.83
	31	0.91
	32	0.98

Figure 27 CPG-DEAE chromatography of BNC GAG a) Run I, ~ 400 µg uronic acid. b) Run II, ~ 600 µg uronic acid.

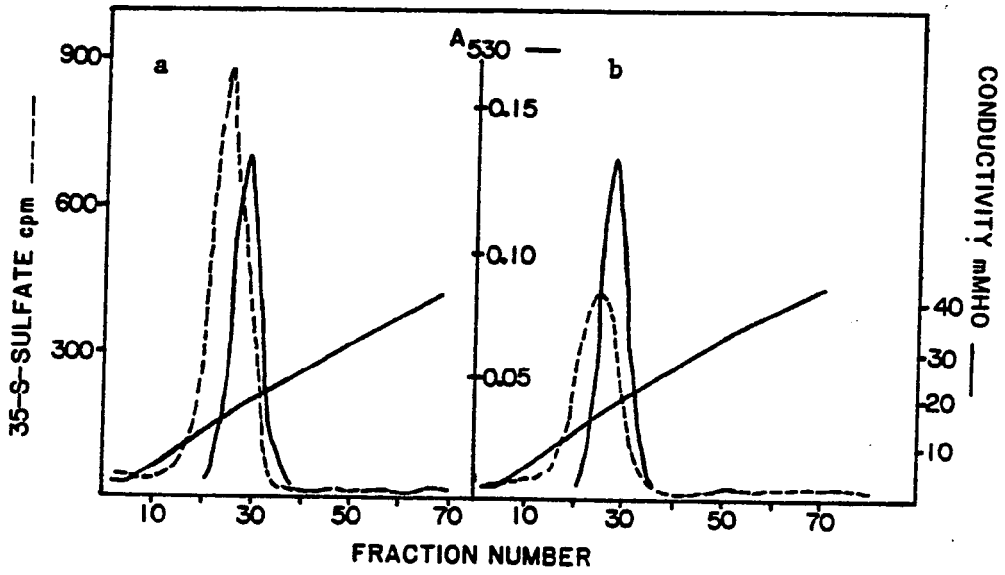

CONDUCTIVITY mMHO
40
20
II
50
30
10
0.25
0.20
0.15
0.10
0.05
FRACTION NUMBER
40
20
I
50
30
10
0.15
0.10
0.05
ABSORBANCE 530 nm

probably reflects the difficulties in accurate sulfate analysis and the increased error when a ratio of two determinations is taken. Nevertheless, for an increase of 2.5 conductivity units from fraction #27 to #30 in Run I, the ratio increases by 0.37. Similarly, in Run II, the ratio increases by 0.36 from fraction #27 to #30, a conductivity increase of 2.5 units. In general, the gradient was such that a change in conductivity of 2 mMHO was equivalent to a change of 0.05 in molarity of NaCl.

CPG-DEAE Chromatography of Mouse ^{35}S-Glycosammoglycans

Although changes in the degree of sulfation of glycosaminoglycans from osteoarthritic cartilage have not been reported, undersulfation of proteoglycans in articular cartilage, as in brachymorphic mice, can have a disastrous effect. As a means of comparing their degree of sulfation, ^{35}S-glycosaminoglycans from normal and osteoarthritic mice were fractionated on a column of CPG-DEAE. An internal standard of BNC glycosaminoglycans was always included. A typical profile (Figure 28) reveals that both STR/N and STR/1N mouse ^{35}S-GAG eluted earlier (0.40 M NaCl and 0.38 M NaCl, respectively) than the BNC GAG (0.46 M NaCl and 0.44 M NaCl) and with a broader peak. ^{35}S-GAG from STR/N and STR/1N mice, ages 3.5 and 16 months, were also analyzed. Results from the 3.5-month-old mice (not shown) were similar to those from the 5-month-old mice, suggesting that the ^{35}S-GAG are sulfated to the same degree in both STR/N and STR/1N at both ages. However, the ^{35}S-GAG from 16-month-old mice of both strains appeared to be different (not shown). These GAG eluted with a broader peak and, in

Figure 28 CPG-DEAE chromatography of ^{35}S-GAG from mouse articular cartilage. Carrier BNC GAGS were detected by uronic acid assay, A_{530}; mouse-GAG detected by cpm ^{35}S. Mice were 5 mo. old. a. STR/N; b. STR/1N.


900
600
300
35-S-SULFATE cpm
a
A530
0.15
0.10
0.05
b
CONDUCTIVITY mMHO
40
30
20
10
10
30
50
70
10
30
50
70
FRACTION NUMBER

relation to the elution position of the BNG GAG, eluted earlier than those from 3.5 and 5-month-old mice. Nevertheless, there was no difference between N and 1N at this age.

In summary, analysis of ^{35}S-glycosaminoglycans from osteoarthritic mouse articular cartilage revealed no differences from those of normal mice with respect to: 1) hydrodynamic size of single chains, 2) sensitivity to chondroitinase AC or ABC, 3) percent C-4-S, 4) level of sulfation.

The Link Proteins

Since the link proteins play a role in the stabilization of PG aggregates and were reported missing in at least one study of aged articular cartilage (Perricone et al., 1977), it was important to determine whether or not they were present in articular cartilage from the osteoarthritic mice.

The presence of the link protein(s) in the ^{125}I-labeled A1 from mouse articular cartilage was demonstrated by using specific immunological procedures, polyacrylamide gel electrophoresis in SDS, and autoradiography. Mouse ^{125}I-A1 (10-20 µl, ~ 50,000 cpm) and BNC ^{125}I-A1 (10-20 µl, ~ 50,000 cpm) were each mixed with 0.5% sodium deoxycholate, 0.25% Nonidet P-40, 0.01 M sodium phosphate, pH 7.5 (100 µl), heated for 3 min. at 100°C, and cooled to room temperature. Control mixtures were prepared in the same way but not heated. Antiserum (R #12) (100 µl of a 1/50 dilution) specific for link proteins 1 and 2 from bovine nasal cartilage was added to the heated and unheated

samples and incubated at room temperature for 1 h. with shaking. S. aureus (100 µl of a 10% w/v suspension in wash buffer, Caterson et al., 1980) was mixed with the antiserum/antigen mixtures, and the incubation was continued at room temperature for 10 min. The cells were pelleted by centrifugation at ~ 1000 x g for 10 min., washed twice with wash buffer (1 ml.), and then heated in 2% SDS, 5% mercaptoethanol (100 µl) at 100°C for 3 min. Finally S. aureus was pelleted, and an aliquot (50 µl) of the supernatant was fractionated by polyacrylamide slab gel electrophoresis in SDS. An autoradiogram of this electrophoretic separation is shown in Figure 29. Mouse ^{125}I-A1 (Figure 29f) shows a protein band in the running gel with a mobility similar to that of link protein 2 from BNC ^{125}I-A1 (Figure 29e). When mouse ^{125}I-A1 was heated with detergents and reacted with the antiserum, only one protein band was eluted from the S. aureus/antibody/antigen complex (Figure 29d). Again, this protein has the same mobility as that of link protein 2 from BNC A1 which had been treated in a similar manner (Figure 29b). Thus, the mouse articular cartilage proteoglycan aggregate contains only one link protein (link protein 2). Since other experiments (Caterson et al., 1980) have shown that this antiserum recognizes native undenatured link proteins, the requirement for heating (Figure 29a,c) suggests that the link protein(s) is sequestered in the unheated proteoglycan aggregates and unavailable for reaction with the antiserum.

When ^{125}I-labelled A1 fractions from STR/N and STR/1N mouse articular cartilage were examined for LP using the above procedure, no differences between the two strains were seen (Figure 30).

Figure 29 The immunoprecipitation of ^{125}I-link proteins from BNC A1 and mouse A1 preparations. ^{125}I-A1 from both sources was preheated at 100°C or reacted directly with antiserum to link proteins. Immunoprecipitated ^{125}I-proteins were absorbed to and eluted from an S. aureus preparation, separated by polyacrylamide gel electrophoresis, and located by autoradiography (details are given in the text). a, BNC ^{125}I-A1, immunoprecipitated; b, BNC ^{125}I-A1, preheated, immunoprecipitated; c, mouse ^{125}I-A1, immunoprecipitated; d, mouse ^{125}I-A1, preheated, immunoprecipitated; e and f, BNC ^{125}I-A1 and mouse ^{125}I-A1, respectively, not immunoprecipitated. T = top of stacker gel.

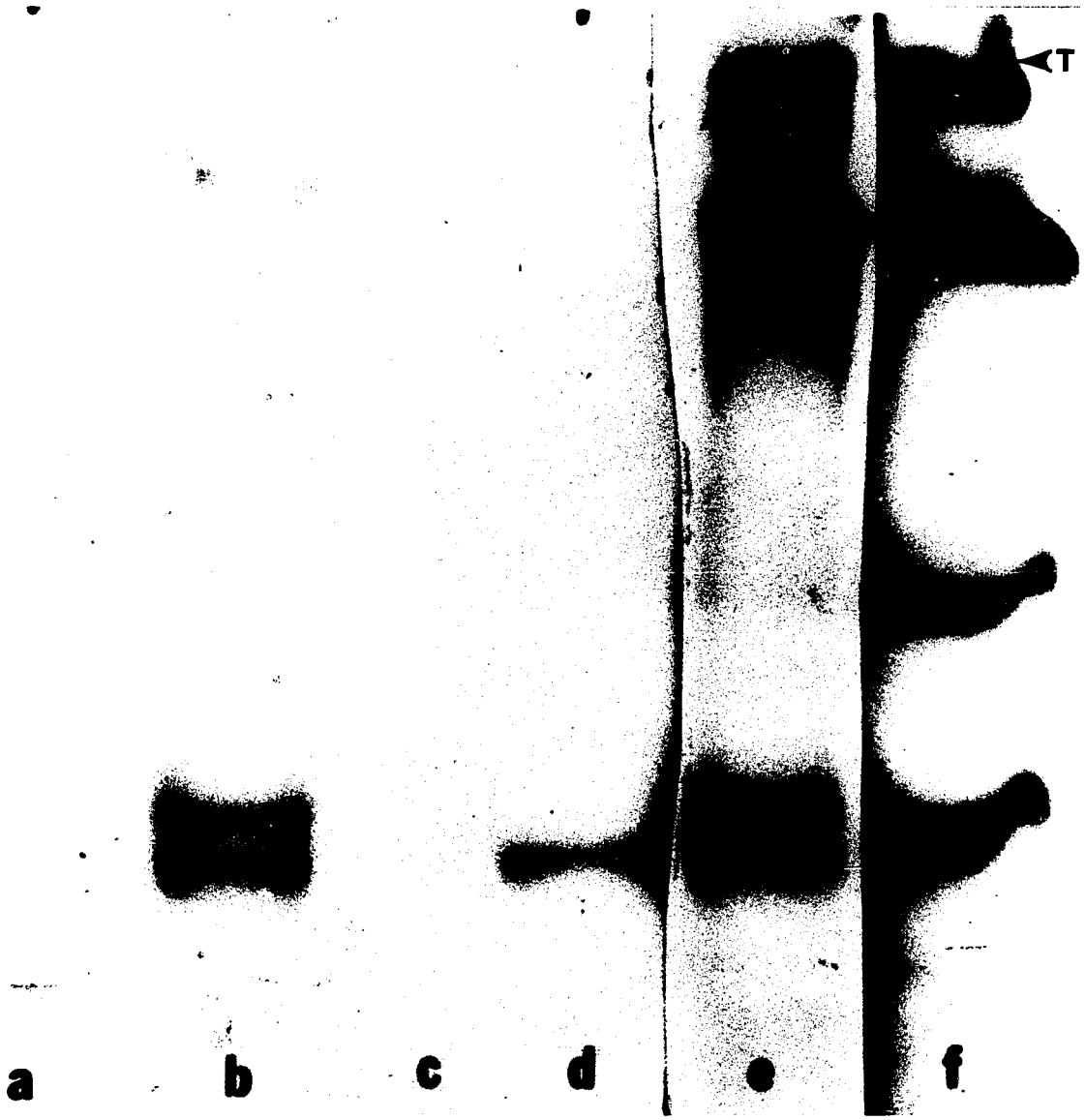
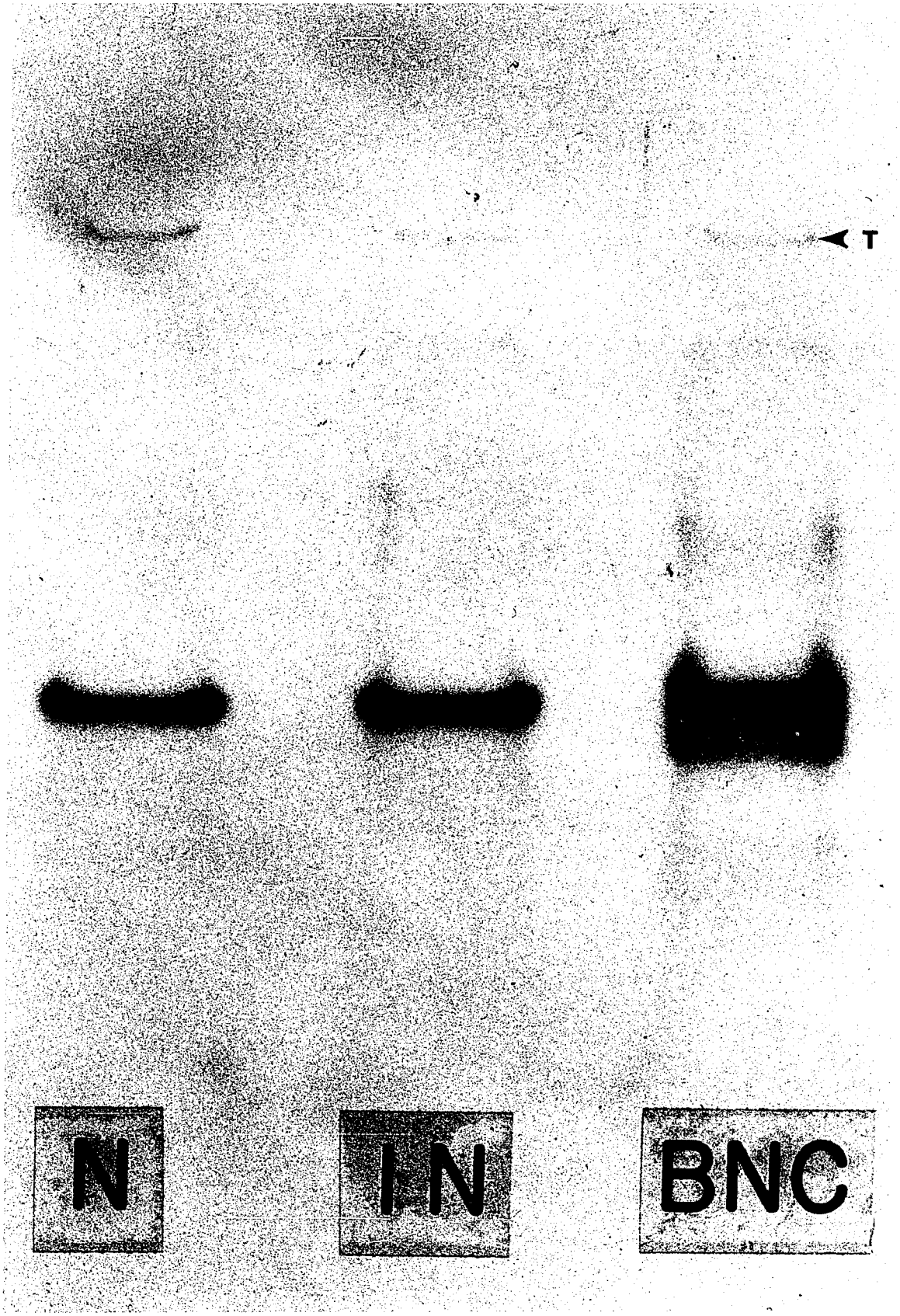

T
a
b
c
d
e
f

Figure 30 Immunoprecipitation of ^{125}I-link proteins from BNC-A1 and mouse A1 preparations from STR/N and STR/1N animals. Conditions same as in Figure 29. T = top of stacker gel.


T
N
I N
BNC

Interestingly, only LP 2 is found in the aggregates from these mice. This may be a characteristic of rodent hyaline cartilage, since LP 2 is the only LP found in the Swarm rat chondrosarcoma (Oegema et al., 1977; Caterson & Baker, 1979; Oegema et al., 1975; Faltz, Reddi, Hascall, Martin, Pita, & Hascall, 1979) and rat epiphysial growth cartilage (Pita et al., 1979).

SUMMARY AND CONCLUSIONS

Airfuge Methodology

The results using BNC PG and BNC extracts show that proteoglycan aggregates and their components can be rapidly isolated using preformed CsCl density gradients in the Beckman Airfuge. In addition, when continuous 206 nm monitoring of gel chromatographic separation is employed, the isolation and characterization of proteoglycans can be accomplished in several days instead of several weeks. In comparison, a microscale technique developed by Pita et al. (1979) for CsCl gradient fractionation of proteoglycan requires a 48-h centrifugation to achieve the equilibrium distribution of proteoglycan. Much shorter times have been needed for sedimentation equilibrium studies of proteins in the Airfuge (Bothwell et al., 1978; Pollet et al., 1979). This study extends the use of the Airfuge to fractionation of proteoglycans in preformed CsCl density gradients in only 4 h. The speed and ease of processing many individual samples in the Airfuge, the improved extraction technique, and the use of specific antibodies made feasible the characterization of mouse articular cartilage proteoglycan from normal and osteoarthritic animals.

Mouse Articular Cartilage Proteoglycans in Normal and Osteoarthritic Mice

Articular cartilage proteoglycans from normal (Swiss or STR/N) and osteoarthritic (STR/1N) mice have been analyzed and compared with respect to several parameters (Table 17). In contrast to many other studies of PG from OA cartilage (Table 1), no major PG abnormality was found in the OA mice.

TABLE 17

SUMMARY OF COMPARISON OF ARTICULAR CARTILAGE PROTEOGLYCANS FROM NORMAL AND OSTEOARTHRITIC MICE

	NORMAL	OSTEOARTHRITIC
Extractability (%)		
0.4 M GuHCl	19 ± 4	20 ± 7
Residue	36 ± 6	26 ± 8 ($p < 0.05$)
Distribution in CsCl density gradients (% in A1)	62 ± 15	70 ± 14 ($p > 0.10$)
Ability to aggregate	OA PG can aggregate as well as normal PG	
Monomer size	Similar ($K_{AV} = 0.46$).	
GAG chain size	Similar ($K_{AV} = 0.65$).	
Sulfation of GAG	GAG elute at similar molarities NaCl from CPG-DEAE.	
% C-4-S		
1-3 mo.	91	94
7-12 mo.	81	76
Link proteins	Only LP 2 in both.	

The hypothesis that PG from OA cartilage could not aggregate (e.g., Palmoski & Brandt, 1976; Perricone et al., 1977; Moskowitz et al., 1979) was prevalant in the literature at the time this mouse study was initiated. The results presented herein demonstrate that PG from OA mice do aggregate despite severe degeneration of the cartilage. That this may be otherwise in other models is possible, however, McDevitt and Muir (1979) reported that the PG from OA dog cartilage could aggregate although there were proportionally fewer aggregates compared to monomer.

McDevitt and Muir (1976) had reported that aggregates from dog OA cartilage were smaller than normal when analyzed in the analytical ultracentrifuge. It was not possible to measure aggregate size directly for mouse PG, but diminished PG monomer size due to a shorter CS-region and/or shorter CS chains could result in diminished aggregate size. The monomer sizes for OA and normal mice were comparable as were the sizes of the CS chains. Decreased HA length could also produce smaller aggregates but the size of HA is difficult to measure in this model.

The increase in low salt extractable PG from OA cartilage reported by McDevitt and Muir (1976) was not found when comparing normal and OA mice in this study. However, a higher proportion of PG was removed by the sequential 4 M GuHCl extraction of OA mouse cartilage as compared with normal mice. This has not been reported for other models and is interesting in view of the unknown nature of the unextractable PG in normal cartilage.

Orkin et al. (1976) found predominately C-4-S in the epiphysis of 5-day-old normal mice and the GAG from these mice were totally digested by chondroitinase AC. Likewise, the GAG from young (1-3 mo.) STR/N, STR/1N, and Swiss mouse cartilage yielded ~ 90% ΔDi-4-S when treated with chondroitinase and were digested equally by Csase AC and ABC. Since older mice (7-12 mo.) of all three strains had 70-80% C-4-S, the OA animals do not appear to be producing higher levels of 4-sulfated PG than normal as suggested by Michelacci et al. (1979) and Mankin and Lippiello (1971).

Elution of bm/bm mouse GAG from DEAE-cellulose (Orkin et al., 1976) suggested that the GAG from these animals were undersulfated. ^{35}S-GAG from bm/bm mice eluted at a molarity of NaCl 0.08 lower than the molarity at which ^{35}S-GAG from normal mice eluted. However, when labelled with ^{3}H, a peak from bm/bm mouse GAG was seen at a molarity of one-half that of normal mouse GAG; no ^{3}H-GAG was seen at this molarity for the normal mice. The ^{35}S-GAG from STR/N and STR/1N elute at similar molarities of NaCl from CPG-DEAE, which would suggest they are sulfated to a similar level. It is possible that labelling with ^{3}H could reveal a much lower sulfated GAG that is not detected in the ^{35}S-experiments.

The absence of link protein(s) in OA cartilage has been reported (Perricone et al., 1977). This would certainly compromise the stability of PG aggregates and possibly reflect more extensive biosynthetic aberrations. There is no evidence to suggest absence or degradation of LP in the OA mouse. It may be, as suggested by Bayliss and Venn (1980), that degradation during isolation was responsible for

Perricone's results. The finding of only LP2 in the mouse cartilage is consistent with other studies of rodent hyaline cartilage (Oegema et al., 1977; Caterson & Baker, 1979; Pita et al., 1979).

The mouse strain, STR/1N, was chosen as an animal model for OA for several reasons. Of special interest was the possibility that OA in these animals might involve a general metabolic (endocrine?) abnormality rather than the mechanical elements of other models. Indeed, it has been proposed that metabolic factors are more important in small animals' joints than in larger animals' joints (Young et al., 1979, p. 257-261).

Despite the involvement of patellar displacement, it is still possible that metabolic factors are important in the OA of STR/1N mice. Furthermore, the absence of a major PG abnormality does not preclude the possibility that a minor PG alteration could be important in the cartilage degeneration in these mice.

Osteoarthritis

It is clear from these results that the articular cartilage proteoglycans from these osteoarthritic mice are not significantly different, at least by the parameters studied, from the proteoglycans of the control mice. Thus, the questions remain: 1. is there any proteoglycan abnormality associated with OA in these animals?, and 2. is the abnormality, if it exists, the cause of the OA? The latter question is probably the more difficult one to answer; collagen, PG,

and chondrocytes in the articular cartilage are so functionally interdependent that the exact sequence of events during the initiation of degeneration is difficult to analyze. But the possibility of a PG abnormality associated with this mouse OA still exists.

Although the water content of the cartilage could not be studied in this mouse model, increase in hydration has been such a consistent finding in other studies, it warrants further consideration. Since distension of the collagen network limits the tendency of the PG to imbibe water, an increase in hydration may reflect an alteration in the bonding of collagen fibers with each other or with other matrix elements. The collagen fibers themselves appear to be composed of normal Type II collagen even in OA cartilage (Muir, 1977; Eyre, McDevitt, Billingham, & Muir, 1980). The nature of the bonding agent between fibers is unknown, although it has been suggested that a PG fraction may be a structural component of the collagen network (Freeman & Meachim, 1979). The existence of at least two populations of PG whose functions are unknown -- the non-aggregating PG (Heinegard & Hascall, 1979) and the low-molecular weight PG described by Heinegard et al. (1981) -- suggests that the search should continue for a PG fraction that is involved in the maintenance of the collagen network. The increase in total extractable PG from the OA mouse cartilage may be relevant to this. It is unknown if the unextractable PG represents a functionally unique class of molecules, although they appear chemically similar to the extractable population (Christner, personal communication).

Two recent studies (Muir & Sandy, 1982; Harmand, Duphil, & Blanquet, 1982) have examined the PG produced by OA and normal chondrocytes in culture. Muir and Sandy studied both cartilage slices and isolated chondrocytes from dog articular cartilage. Cartilage slices were pre-labelled in vitro with ^{35}S-SO_4. The OA cartilage released more ^{35}S-PG into the medium than the normal cartilage did. The results suggested that this enhanced release was due to increased degradation of the newly synthesized PG by the OA cells. Whether in slices or isolated, the OA cells produced an immature type of PG, rich in CS, and abnormally large amounts of a relatively low molecular weight PG (K_{AV} = 0.75 on Sepharose CL-2B). One interesting result was that OA chondrocytes in cartilage slices were more active than normal cells in slices, but isolated chondrocytes were less active than isolated normal cells. Whether this is a general effect related to removal of matrix or an effect of a specific factor in the OA matrix is not known.

Harmand et al. analyzed the PG produced by cartilage explants of human femoral head cartilage. In their system, there was no difference in the sulfated macromolecules in the cell matrix and medium, but the cells themselves accumulated, intracellularly, GAG-peptides (MW = 60,000) and a nonaggregating PG of K_{AV} = 0.33 on Sepharose CL-2B. The GAG-peptides could result from PG degradation since the GAG are similar to those from the intact PG. However, kinetic studies suggested that degradation was comparable in both normal and OA cells. Thus, these investigators suggest the defect is in the control of synthesis rather than degradation. The intracellular, nonaggregating PG did not

seem to be secreted and may be present in normal cells, but at lower levels.

Results from both of the above studies point to an imbalance in the synthesis and degradation of PG in OA chondrocytes. Unfortunately, the controls on these processes are not well understood. Furthermore, although PG are isolated as aggregates soon after synthesis and aggregation is known to occur extracellularly (Kimura, Hardingham, Hascall, & Solursh, 1979), little is known about the mechanism by which these events occur. What processes occur between the completion of synthesis in the Golgi and the final stable aggregation of all components? Oegema (1980) has suggested that in some human OA cartilages there is a delayed conversion from an HA-noninteractive precursor to a form that can interact with HA. The accumulation of various sulfated macromolecules inside OA chondrocytes may be a reflection of some abnormality of normal processing and transportation of newly synthesized PG to the extracellular matrix. Whether or not this proves to be the cause of OA, analysis of OA chondrocytes may shed light on the mechanisms by which normal cells maintain the cartilage matrix.

There are data that suggest hormonal effects are involved in the development of OA in humans (Peyron, 1979). The prevalence of OA, as assayed by X-ray of hands and feet, tends to be slightly higher in men under the age of 45; to be roughly equal between the sexes around 45-55; but is markedly increased for women over 55. Multiple joint involvement is more frequent in females than in males, the difference

being more noticeable at the older ages. In general, the disease tends to become more widespread and more severe in women than in men.

Even though women are more susceptible to OA, they may be somewhat spared until menopause, suggesting a protective effect of estrogens. That estrogen can protect against the onset of OA has some limited support from studies in mice. In several strains prone to osteoarthritis, the arthropathy predominates in males in whom its prevalance can be reduced by orchiectomy (Silberberg, Thomasson, & Silberberg, 1958) and by administration of estrogens (Silberberg & Silberberg, 1963). However, in other predisposed strains (e.g., STR/1N) the males are not rescued by castration (Sokoloff, 1961; Sokoloff et al., 1965).

Since the results to date in humans and mice do not provide a clear answer, the role of hormones in the development of OA should be studied further. The availability of refined techniques, e.g., RIA, for measuring the levels of many hormones may enhance the accuracy of new studies. Furthermore, with regard to the STR/1N mice, there is evidence that suggests an hypothalamic lesion in these animals (Silverstein, Sokoloff, Mickelsen, & Jay, 1961). It would be interesting to examine, by RIA now available, the levels of growth hormone and somatomedins in these mouse strains. [A study of women at the time of menopause with early OA showed a level of circulating growth hormone more than twice that of control subjects with no joint abnormality (Peyron, 1979).]

Finally, mention should be made of the probable clinical heterogeneity of osteoarthritis. Can it be said that OA of the hip is the

same disease as OA of the knee, or finger joints, or vertebrae? It is probable that there are many factors involved -- local articular factors related to the configuration and mechanical forces exerted on the joint and more generalized metabolic properties of the articular tissues. The contribution of each factor may vary in various animal models of OA as well as in the human condition. Nevertheless, if some common pathway to cartilage degeneration can be defined, there is hope of finding a means to halt, or at least retard, the process.

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