Isolation of Dermatan Sulfate Proteoglycans from Mature Bovine Articular Cartilages*

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Lawrence C. Rosenberg, Haing U. Choi, Lih-Heng Tang, Thomas L. Johnson, and Subhash Pal
From the Orthopedic Research Laboratories, Montefiore Medical Center, Bronx, New York 10467

Carolyn Webber, Agnes Reiner, and A. Robin Poole
From the Joint Diseases Laboratory, Shriners Hospital for Crippled Children, Montreal, Quebec H3G 1A6, Canada

Two species of dermatan sulfate proteoglycans, called DS-PGI and DS-PGII, have been isolated from mature bovine articular cartilages. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis at low ionic strength in 0.01 M phosphate the dermatan sulfate proteoglycans appeared as a single polydisperse species whose molecular weight ranged from 80,000 to 140,000. The dermatan sulfate proteoglycans eluted as a single peak on Sepharose CL-4B chromatography in 4 M guanidine hydrochloride and showed no tendency to separate into two components. Following chondroitinase AC and ABC digestion, a core protein was obtained whose molecular weight was 45,000. However, what appeared to be a single dermatan sulfate proteoglycan was consistently separated into two species of distinctly different mobilities by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at high ionic strength in 0.375 M Tris. The molecular weight of the smaller species (DS-PGII) ranged from 87,000 to 120,000. The molecular weight of the larger species (DS-PGI) ranged from 165,000 to 285,000. DS-PGI self-associates in 0.375 M Tris, while DS-PGII does not. This phenomenon was exploited to separate DS-PGI and DS-PGII by preparative electrophoresis on 5 to 20% gradient slab gels.

The immunological identities of the individual species, DS-PGI and DS-PGII, were examined by enzymelinked immunosorbent assay using polyclonal antiserum to cartilage-specific proteoglycan monomer from bovine articular cartilage and polyclonal and monoclonal antibodies to DS-PGII. The polyclonal antiserum to cartilage-specific proteoglycan monomer did not react with DS-PGI or DS-PGII, indicating that DS-PGI and DS-PGII possess different core proteins from cartilage-specific proteoglycan monomer. Polyclonal and monoclonal antibodies raised against the mixture of DS-PGI and DS-PGII reacted strongly with DS-PGI, but weakly or not at all with DS-PGII. These results suggest that DS-PGI and DS-PGII possess different core proteins and may represent two different species of dermatan sulfate proteoglycans.

Dermatan sulfate-containing proteoglycans are widely distributed in the extracellular matrix of blood vessel wall (1-11), skin (12-16), tendon (17), sclera (18-21), and a variety of other tissues (22). Dermatan sulfate proteoglycans have not, to our knowledge, been demonstrated in the extracellular matrix of mature or aging articular cartilages. Articular cartilages contain mainly cartilage-specific proteoglycan monomers, which consist of chondroitin sulfate and keratan sulfate bound to a protein core. Most of the cartilage-specific proteoglycan exists in the form of proteoglycan aggregates of huge size, formed by the noncovalent association of proteoglycan monomers, link protein, and hyaluronic acid (23-35). Compared with cartilage-specific proteoglycans, dermatan sulfate proteoglycan monomers are usually much smaller in size and do not bind to hyaluronic acid to form large stable aggregates, but self-associate to form relatively small, unstable aggregates (19).

Dermatan sulfate proteoglycans are also important components of the extracellular matrix of undifferentiated mesenchymal tissue, present in the developing limb before the formation of a cartilaginous model of the developing bone. Prior to the advent of chondrogenesis, the developing limb bud consists of a core of mesenchyme covered by ectoderm. The extracellular matrix of this undifferentiated mesenchyme contains mainly a low molecular weight dermatan sulfate proteoglycan (36-38). With the advent of chondrogenesis, synthesis of the cartilage-specific proteoglycan suddenly increases, and synthesis of the dermatan cartilage-specific mesenchymal proteoglycan is relatively decreased (36-38).

Because of the difficulty in isolating the mesenchymal dermatan sulfate proteoglycan from undifferentiated mesenchymal tissue in amounts sufficient for its characterization, little is known about its structure and properties. In a recent study, we found that small amounts of a dermatan sulfate proteoglycan were present in bovine fetal epiphyseal cartilage (39). The molecular weight of the isolated dermatan sulfate proteoglycan ranged from 80,000 to 140,000, and it possessed a core protein whose molecular weight was approximately 45,000. An antiserum was raised against the dermatan sulfate proteoglycan and its localization throughout the extracellular matrix of fetal epiphyseal cartilage was demonstrated (39). However, the ratio by weight of cartilage-specific proteoglycan to dermatan sulfate proteoglycan in near-term fetal epiphyseal cartilage was approximately 500:1, and only extremely small amounts of the species could be isolated from several large bovine fetuses.

In the course of our studies, we examined bovine articular cartilages, to determine whether this tissue might provide a useful source of dermatan sulfate proteoglycans. The results were surprising. Aging bovine articular cartilages contained

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over 10 times as much dermatan sulfate proteoglycan, relative to cartilage-specific proteoglycan, as near-term fetal epiphyseal cartilage. Moreover, the aging articular cartilages contained two species of dermatan sulfate proteoglycans, called DS-PGI and DS-PGII, in amounts sufficient for their isolation and characterization.

In this report, we describe the development of methods for the isolation of DS-PGI and DS-PGII and an analysis of their immunological identities. DS-PGI appears to self-associate while DS-PGII does not. Neither DS-PGI nor DS-PGII is immunologically related to cartilage-specific proteoglycan, from bovine articular cartilage. Polyclonal and monoclonal antibodies which react strongly with DS-PGII react weakly or not at all with DS-PGI. DS-PGI and DS-PGII appear to represent two different species of dermatan sulfate proteoglycans.

EXPERIMENTAL PROCEDURES

Materials—GdmCl "absolute grade" was from Research Plus Laboratories, CsCl "O.D. grade" was from Gallard-Schlesinger, Mes was from Aldrich. PMSF and iodoacetamide were from Sigma. Chondroitinases AC and ABC, chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate were from Miles Research Laboratories. Glycosaminoglycan standards were also provided by Drs. Martin Mathews and J. A. Cifonelli of the University of Chicago (Contract NO1-AM-5-2285 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health).

Isolation of Proteoglycans—Fresh wet bovine articular cartilage from 3- to 4-year-old cows was shaved from the occipital condyles, finely diced into 1-mm cubes, and added to ice-cold 4 M GdmCl, 0.05 M EDTA, 0.15 M sodium acetate, pH 7.0, containing 0.005 M concentrations of benzamide hydrochloride, PMSF, and iodoacetamide as protease inhibitors. The cartilage was extracted by slow stirring at 5 °C for 24 h. Extracts were filtered, then dialyzed against 20 volumes of 0.15 M sodium acetate, 0.05 M EDTA, pH 7, containing 0.005 M concentrations of the protease inhibitors at 5 °C for 16 h. Equilibrium density gradient centrifugation under associative conditions was carried out in 3.5 M CsCl at 5 °C for 50 h at 40,000 rpm. The gradient was divided into six equal fractions, called A1 through A6 (40). Aliquots of these fractions were dialyzed against 0.15 M sodium acetate, pH 6.3, precipitated with 3 volumes of ethanol, washed 3 times with ethanol and ether, and dried in a vacuum. The chondroitin sulfate and dermatan sulfate contents of the fractions from the associative gradient were determined using chondroitinases AC and ABC. Fractions A2, A3, and A4 which contained dermatan sulfate were further fractionated by equilibrium density gradient centrifugation under associative conditions in 4 M GdmCl, 3 M CsCl, 0.05 M EDTA, 0.15 M sodium acetate, pH 7, containing 0.005 M concentrations of the protease inhibitors at 40,000 rpm for 60 h at 5 °C. The dissociative gradient was divided into six equal fractions called D1 through D6 (40). Aliquots of these fractions were dialyzed against 0.15 M sodium acetate, pH 6.3, precipitated with 3 volumes of ethanol, washed 3 times with ethanol and ether, and dried in a vacuum. The chondroitin sulfate and dermatan sulfate contents of these fractions were then determined.

Chromatography on DEAE-Sepharose CL-4B—The following procedures were carried out at 5 °C. Dermatan cartilage-specific fractions from the dissociative gradient were dialyzed against 6 M urea, 0.025 M Tris, pH 6.5, containing 0.901 M concentrations of the protease inhibitors, then against the same solvent without the protease inhibitors. The sample was applied to a 2.7 -52-cm DEAE-Sepharose CL-4B column equilibrated in the same solvent. Approximately 150 mg of sample in 30 ml of buffer was loaded on each column. The column was eluted with a linear gradient of 0 to 1 M NaCl in 6 M urea, 0.025 M Tris, pH 6.5, at a flow rate of 22 ml/h. Fractions of approximately 10 ml were collected and assayed for uronate (carbohydrate), hexose (ammonia), and protein (A280). Fractions containing proteoglycan were pooled, dialyzed against 0.15 M sodium acetate, pH 6.3, precipitated with 3 volumes of ethanol, and collected by centrifugation, washed with ethanol and then ether, and dried.

Isolation of Glycosaminoglycan Chains—Isolate proteoglycans were degraded in 0.5 M NaOH, 1 M NaBH4, at 45 °C for 24 h. The solution was brought to pH 5 with 6 N acetic acid in an ice bath, then dialyzed against 0.1 N NaCl. The glycosaminoglycan chains were then purified by chromatography on DEAE-Sepharose using a 0.1 to 3 M NaCl linear gradient. The glycosaminoglycans, which eluted at 0.05 M NaCl, were dialyzed against 0.15 M sodium acetate, pH 6.5, precipitated with 3 volumes of ethanol, collected by centrifugation, washed in ethanol and ether, and dried.

Preparation of Proteoglycan Core Proteins—Proteoglycan core proteins were prepared by digesting proteoglycans with chondroitinase AC or chondroitinase ABC in the presence of protease inhibitors under conditions described by Okie et al. (41, 42). Chondroitinase AC digestions were carried out in 0.03 M sodium acetate, 0.1 M Tris, pH 7.4, containing 0.36 mM peptatin, 5 mM PMSF, 10 mM N-ethylmaleimide, and 10 mM EDTA. Chondroitinase ABC digestions were carried out in the same solution at pH 5. Approximately 1 mg of proteoglycan was dissolved in 1 ml of the digestion mixture, to which 0.05 unit of chondroitinase had been added. The digestion was carried out for 24 h at 37 °C. The enzymes were inactivated by boiling for 2 min. The solutions were then dialyzed against distilled water and lyophilized. The molecular weights of the core proteins were then determined by SDS-PAGE using analytical 4 to 20% acrylamide gels. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—SDS-PAGE on 4% acrylamide disc gels was carried out in conventional low ionic strength 0.1 M sodium phosphate buffer, pH 7, containing 0.1% SDS and 0.1% β-mercaptoethanol, as described previously (43, 44).

Analytical SDS-PAGE on 4 to 20% acrylamide gradient slab gels was carried out at high ionic strength in 0.375 M Tris, 0.1% SDS, pH 8.8, as described by Laemmli and Favre (45). A 4 to 20% acrylamide gradient gel was prepared in 0.375 M Tris, 0.1% SDS, pH 8.8, to which was added a 3.6% acrylamide stacking gel in 0.125 M Tris, pH 6.8. The running buffer was 0.1% SDS, 0.05 M sodium, 0.05 M Tris, pH 8.4. The sample buffer contained 1% SDS, 1% β-mercaptoethanol, 0.0006% bromphenol blue, and 16% glycine in 0.08 M Tris, pH 6.8. SDS-PAGE was carried out at 200 V for 4 h at 12 °C. The gels were removed and gently shaken overnight in 10 volumes of 45% methanol containing 5.5% acetic acid. The gels were stained for 15 min in 0.2% toluidine blue in 0.1 N acetic acid. The gels were destained in two changes of 3% acetic acid (2 h) and in two changes of water and then photographed.

Molecular weights of the isolated proteoglycans were calculated in the case of disc gel, from plots of the logarithms of the molecular weights of the standards versus their mobilities. In the case of the gradient slab gels, molecular weights were determined from plots of the molecular weights of the standards versus the logarithms of the gel concentrations at the location of the standard or unknown sample, as described by Poduslo and Rodbard (46) and by Lambin (47).

Separation of DS-PGI and DS-PGII by Preparative SDS-PAGE in 0.375 M Tris—From the mixture of DS-PGI and DS-PGII obtained
Dermatan Sulfate Proteoglycans

following gel chromatography on Sepharose CL-4B in 4 M GdmCl, DS-PGII and DS-PGIII were separated and isolated to homogeneity by preparative SDS-PAGE in 0.375 M Tris. The same conditions were used as for analytical SDS-PAGE, except that a 6-mm 5 to 20% gel was prepared in a Beckman Model E analytical ultracentrifuge with an AnE rotor, using long cells with 30-mm double sector centerpieces, as previously described (32). Runs were made in 0.15 M NaCl, 0.01 M Mes, pH 7, at 20 °C. All additions of solvent to dry samples and dilutions were made with water. Schlieren patterns were photographed on Kodak technical plates and read with a Nikon microphotometer. Extrapolations to \( s_0^0 \) were made from a least-squares curve fit of plots of \( s \) against \( c \) as previously described (32).

Preparation of Antiserum—Antiserum S27 to cartilage-specific proteoglycan monomer (AID1) from mature bovine articular cartilage was prepared and characterized as described previously (58, 59). Antiserum R166 was prepared to the mixture of dermatan sulfate proteoglycans (DS-PGII and DS-PGIII) isolated following gel chromatography on Sepharose CL-4B in 4 M GdmCl. Fractions were taken which contained only the dermatan sulfate proteoglycans and no cartilage-specific proteoglycan, after early fractions containing trace amounts of cartilage-specific proteoglycan were identified by ELISA analyses. This involved the assay as described with the replacement of alkaline phosphatase-labeled pig anti-mouse IgG with alkaline phosphatase-labeled pig anti-rabbit IgG (Fab'2) preparations. The latter were prepared as described (59). An alkaline phosphatase (Sigma type VII-S)-labeled pig IgG anti-mouse IgG and anti-rabbit IgG (Fab'2) were used for these immunoglobulins. Concentrated pig antibody IgG as a 50% saturated ammonium sulfate precipitate was labeled with alkaline phosphatase according to the Sigma technical bulletin for this preparation. Activity of alkaline phosphatase was determined by the addition of 50 \( \mu \)l of the substrate solution consisting of 5 ml of 9.65% diethanolamine buffer, pH 9.8, with 24 \( \mu \)mol MgCl\(_2\) and 6.73 \( \mu \)mol nitrophenyl disodium orthophosphate. After 4 h at 37°C and 14 h at 4°C, the absorbance at 405 nm was recorded with a Titertek Multiscan Plate Reader.

Immunoglobulin class and subclass of monoclonal antibody were determined with class and subclass rabbit antisera (Miles) using ELISA analyses. This involved the assay as described with the replacement of alkaline phosphatase-labeled pig anti-mouse IgG immuno-globulin serum with rabbit antisera followed by alkaline phosphatase-labeled pig anti-rabbit IgG (Fab'2) preparations. These were closed 10 times by limitation of dilution. The closed cells of one hybridomas to DS-PGII were gradually adapted to medium without HAT, expanded, and inoculated intraperitoneally into BALB/c mice. Aseptic fluid was collected at 10 to 14 days.

Immunogen—IgG F(ab')\(_2 \) antibody subunits were prepared as described (60).

Immunoblotting—Electroblotting onto nitrocellulose using BioRad Trans Blot equipment was at 0.3 A for 16 h, and subsequent washing of the nitrocellulose was as previously described (64). Nitrocellulose was then incubated for 4 h at 37°C with immune and
Dermatan sulfate-containing proteoglycans were isolated from mature bovine articular cartilages by the procedure shown in Diagram 1. Proteoglycans were extracted in 4 M GdmCl containing protease inhibitors, then fractionated by equilibrium density gradient centrifugation under associative conditions. Fractions A1 through A6 were examined for the presence of dermatan sulfate using chondroitinas AC and ABC. The yields and chemical compositions of fractions A1 through A6 from a representative associative density gradient are shown at the top of Table I. Dermatan sulfate was not detectable in fraction A1. Fractions A5 and A6 contained small amounts of dermatan sulfate. Fractions A2, A3, and A4 contained the highest dermatan sulfate contents, whereas dermatan sulfate accounted for 5.3 to 14.8% of the dry weight of a particular fraction.

The distribution of the dermatan sulfate proteoglycan in a representative associative density gradient is clearly demonstrated in a histogram which shows the percentage of the total dermatan sulfate in the entire associative gradient recovered in each fraction (Fig. 1). Approximately 90% of the chondroitin sulfate is associated with the cartilage-specific proteoglycan recovered in fraction A1. Fraction A1 contains no dermatan sulfate. Fractions A2, A3, and A4 contain approximately 80% of the dermatan sulfate proteoglycan present in associative gradients.

The dermatan sulfate-containing proteoglycan present in fractions A2, A3, and A4 was much smaller in size than the cartilage-specific proteoglycan monomer and aggregate present in fraction A1 and could be easily distinguished from these species in sedimentation velocity experiments in the analytical ultracentrifuge. Fig. 2, A and B, shows schlieren patterns of fraction A1. Fraction A1 contains a proteoglycan monomer with a sedimentation coefficient (s20,w) of 18.7 S and an aggregate with a sedimentation coefficient of 109 S, similar to previously described species from mature bovine articular cartilage (32). The concentration dependences of the sedimentation coefficients of the cartilage-specific proteoglycan aggregate and monomer are shown in curves 1 and 2 of Fig. 3.

Fractions A2, A3, and A4 contain mainly a unimodal component with a sedimentation coefficient of approximately 5 S. For example, the arrow in Fig. 2C shows this component in fraction A2. A shoulder of more rapidly sedimenting material, which is contaminating cartilage-specific proteoglycan monomer, is also clearly demonstrated at early times after reaching speed. The arrow in Fig. 2D shows the 5 S component in fraction A3. The concentration dependences of sedimentation coefficients of these components are shown in curves 3 and 4 of Fig. 3. None of the 5 S component is detectable in fraction A1 (Fig. 2, A and B).

The dermatan sulfate proteoglycan was also demonstrated in the associative gradient fractions by SDS-PAGE on 4% disc gels, which were stained with toluidine blue. The dermatan sulfate proteoglycan and the cartilage proteoglycans stain weakly with Coomassie blue, but stain avidly with cationic dyes such as toluidine blue. Fig. 4 shows the appearance of fractions A1 through A6 on 4% disc gels stained with toluidine blue after SDS-PAGE. The dermatan sulfate pro-

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**Results and Discussion**

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/ml)</td>
<td>1.60</td>
<td>1.53</td>
<td>1.47</td>
<td>1.42</td>
<td>1.37</td>
<td>1.33</td>
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<tr>
<td>Yield (g/g)</td>
<td>0.618</td>
<td>0.650</td>
<td>0.033</td>
<td>0.035</td>
<td>0.082</td>
<td>0.182</td>
</tr>
<tr>
<td>Uronate (%)</td>
<td>16.5</td>
<td>12.2</td>
<td>9.3</td>
<td>5.9</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Hexose</td>
<td>11.3</td>
<td>6.0</td>
<td>5.8</td>
<td>5.2</td>
<td>4.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Sialate</td>
<td>1.8</td>
<td>0.65</td>
<td>0.65</td>
<td>0.79</td>
<td>0.81</td>
<td>0.77</td>
</tr>
<tr>
<td>Protein</td>
<td>10.9</td>
<td>27.3</td>
<td>35.5</td>
<td>47.3</td>
<td>54.5</td>
<td>65.0</td>
</tr>
<tr>
<td>Dermatan sulfate*</td>
<td>0</td>
<td>11.1</td>
<td>11.9</td>
<td>5.9</td>
<td>3.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Chondroitin sulfate*</td>
<td>41.9</td>
<td>30.3</td>
<td>18.6</td>
<td>10.4</td>
<td>3.9</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Determined using chondroitinas AC and ABC.

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1. Extract fresh wet cartilage in 4 M GdmCl, 0.05 M EDTA, 0.15 M sodium acetate, pH 7, containing protease inhibitors at 5 °C for 24 h.
2. Dialyze extract against 20 volumes of 0.15 M sodium acetate, 0.05 M EDTA, pH 7, containing protease inhibitors, for 16 h at 5 °C.
3. Equilibrium density gradient centrifugation under associative conditions in 3.5 M CsCl, at 40,000 rpm for 50 h at 5 °C. Cut fractions A1 to A6.
4. Determine dermatan sulfate content of fractions A1 to A6 using chondroitinas AC and ABC. Pool the dermatan sulfate-containing fractions.
5. Equilibrium density gradient centrifugation under dissociative conditions of dermatan sulfate-containing fractions in 3.5 M CsCl, 4 M GdmCl, at 40,000 rpm for 65 h at 5 °C. Cut fractions D1 to D6.
6. Determine dermatan sulfate content of fractions D1 to D6 using chondroitinas AC and ABC.
7. Chromatography on DEAE-Sephacel in 6 M urea, 0.025 M Tris, pH 6.5.
8. Chromatography on Sepharose CL-4B in 4 M GdmCl.
9. Preparative SDS-PAGE on 5 to 20% gradient slab gels.

**Diagram 1. Isolation of dermatan sulfate proteoglycans from mature bovine articular cartilages.**

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**Fig. 1. Percentage of chondroitin sulfate (lined bars) and dermatan sulfate (solid bars) recovered in fractions A1 through A6 from mature bovine articular cartilage.**
Dermatan Sulfate Proteoglycans

Fig. 2. Sedimentation velocity patterns of the proteoglycan species present in fractions A1, A2, and A3 from mature bovine articular cartilage. The direction of sedimentation is from left to right. A, fraction A1 at concentrations of 2.98 mg/ml (top) and 2.39 mg/ml (bottom) at 24 min after reaching speed. The faster moving peak is the cartilage-specific proteoglycan aggregate (arrow) whose sedimentation coefficient is 109 S. B, fraction A1 at 104 min after reaching speed. The major component is the cartilage-specific proteoglycan monomer whose sedimentation coefficient is 18.7 S. None of the 5 S component is detectable in fraction A1. C, fraction A2 at concentrations of 3.00 mg/ml (top) and 2.44 mg/ml (bottom) at 32 min after reaching speed. The sedimentation coefficient of the major component is 4.9 S. D, fraction A3 at concentrations of 3.06 mg/ml (top) and 2.56 mg/ml (bottom) at 56 min after reaching speed. The sedimentation coefficient of the major component (arrow) is 5.0 S.

Fig. 3. Concentration dependence of sedimentation coefficients of the proteoglycan species present in fractions A1, A2, and A3 from mature bovine articular cartilage. Curve 1, proteoglycan aggregate in fraction A1; curve 2, cartilage-specific proteoglycan monomer in fraction A1; curve 3, major component in fraction A3 whose sedimentation coefficient \( (S_{20,w}) \) is 5.0 S; curve 4, major component in fraction A2 whose sedimentation coefficient is 4.9 S; curve 5, the low molecular weight proteoglycan isolated from fractions A2, A3, and A4 by chromatography on DEAE-Sephacel and gel chromatography.

The dermatan sulfate proteoglycan appears as a broad, heavily stained band. On SDS-PAGE at low ionic strength in 0.01 M phosphate, the dermatan sulfate-containing proteoglycan appears as a polydisperse species whose \( M_r \) ranges from 80,000 to 140,000 under reducing and nonreducing conditions.

Isolation of the Dermatan Sulfate-containing Proteoglycan—As noted above, fractions A2, A3, and A4 contain approximately 80% of the dermatan sulfate present in an associative gradient. Fractions A5 and A6 usually contain less than 20% of the dermatan sulfate, and the dermatan sulfate proteoglycan in fractions A5 and A6 is mixed with relatively large amounts of cartilage matrix proteins and collagen. Since one of the objectives of this work was to isolate the dermatan sulfate proteoglycan to homogeneity and prepare monospecific antibodies to this species, fractions A2, A3, and A4 were pooled and used to isolate the dermatan sulfate proteoglycan.

The dermatan sulfate proteoglycan in fractions A2, A3, and A4 was further purified by equilibrium density gradient centrifugation under dissociative conditions. The dermatan sulfate contents of fractions D1 through D6 were determined using chondroitinases AC and ABC. The yields and chemical compositions of fractions D1 through D6 from a representative dissociative gradient are given at the bottom of Table I. Fractions D1 and D2 are relatively small in amount and consist mainly of cartilage-specific proteoglycan monomer originally present in A2. Fractions D3 to D6 contain over 90% of the dermatan sulfate. The dermatan sulfate proteoglycan in these fractions was further purified by chromatography on DEAE-Sephacel in 6 M urea (Fig. 5), followed by chromatography on Sepharose CL-4B in 4 M GdmCl (Figs. 6 and 7). When the dermatan sulfate proteoglycan recovered from DEAE-Sephacel was initially chromatographed on Sepharose CL-4B in 4 M GdmCl, a small amount of contaminating cartilage-specific proteoglycan, whose identity was established by ELISA using an antiserum to cartilage-specific proteoglycan, was demonstrated in the leading edge of the chromatogram shown in Fig. 6. The dermatan sulfate proteoglycan was recycled once or twice on Sepharose CL-4B until a single, unimodal peak was obtained (Fig. 7) and showed no tendency to separate into two components. Fractions from the Sepharose CL-4B column were pooled as indicated in Fig. 7 and recovered. On SDS-PAGE at low ionic strength in 0.01 M phosphate, the dermatan sulfate-containing proteoglycan again appeared as a polydisperse species whose \( M_r \), ranged
Dermatan Sulfate Proteoglycans

I.

**Fig. 4.** Toluidine blue-stained 4% disc gels of fractions A1 to A6 from mature bovine articular cartilage. The A1 fraction shows the cartilage-specific proteoglycan at the top of the gel, but no low molecular weight proteoglycan of faster mobility. Fractions A2, A3, and A4 show the dermatan sulfate proteoglycan, which appears as a broad, heavily stained band whose $M_r$ ranges from 80,000 to 140,000 under reducing or nonreducing conditions.

**Fig. 5.** Chromatography of the dermatan sulfate proteoglycan on DEAE-Sephacel in 6 M urea. The proteoglycan was eluted with a linear gradient of 0 to 1 M NaCl and pooled as indicated by the horizontal bar at the top of the figure. from 80,000 to 140,000 under reducing or nonreducing conditions. The chemical composition of the isolated proteoglycan is shown in Table II. The isolated proteoglycan contains approximately 19% dermatan sulfate, based either on analyses using chondroitinases AC and ABC or on determinations of the amount of uronic acid lost after periodate degradation.

The presence of dermatan sulfate in the isolated proteoglycan was also demonstrated by analyses of the chemical composition of its glycosaminoglycan chains. The isolated proteoglycan was subjected to alkaline borohydride degradation, and the glycosaminoglycans were isolated by chromatography on DEAE-Sephacel. The chemical composition of the isolated glycosaminoglycan chains is shown in Table II. The presence of dermatan sulfate in the isolated glycosaminoglycans is unequivocally demonstrated by analyses using chondroitinases AC and ABC and by the decrease in uronic acid content following periodate degradation.

Glycosaminoglycans were removed from the isolated proteoglycan by chondroitinase digestion, and the molecular weight of the core protein was determined by SDS-PAGE. The isolated proteoglycan was digested with chondroitinase AC or with chondroitinase ABC in the presence of protease inhibitors under conditions described by Oike et al. (41, 42). Fig. 8 shows the appearance on a 4 to 20% gradient slab gel.
Chemical composition of the dermatan sulfate proteoglycans and their glycosaminoglycans

<table>
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<tr>
<th>GAG*</th>
<th>DS-PGIb</th>
<th>DS-PGIIb</th>
<th>DS-PGF</th>
<th>DS-PGIIB</th>
</tr>
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<tr>
<td>% dry wt</td>
<td>μmol/μmol protein†</td>
<td>% dry wt</td>
<td>μmol/μmol protein†</td>
<td></td>
</tr>
<tr>
<td>Uronate (carbazole)</td>
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<td>14.6</td>
<td>84.7</td>
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<tr>
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<td>56.2</td>
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<tr>
<td>Dermatan sulfatec</td>
<td>43.3</td>
<td>19.4</td>
<td>47.6</td>
<td>36.4</td>
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<tr>
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<td>26.5</td>
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<td>43.7</td>
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<tr>
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<td>39.4</td>
<td>42.7</td>
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</tbody>
</table>

(DS x 100)/(DS + CS)† | 44.4 | 45.6 | 45.6 | 45.6 | 48.8 |

*Glycosaminoglycans isolated from the mixture of dermatan sulfate proteoglycans (DS-PGI and DS-PGII) isolated following chromatography on Sepharose CL-4B in 4 M GdmCl.

†Composition of the mixture of dermatan sulfate proteoglycans (DS-PGI and DS-PGII) isolated following chromatography on Sepharose CL-4B in 4 M GdmCl.

‡Percentage of the total glycosaminoglycan which is dermatan sulfate. DS is the amount of dermatan sulfate determined by the chondroitinase analyses. CS is the amount of chondroitin sulfate determined by chondroitinase AC analysis.

§Determined by chondroitin AC digestion and thiobarbituric acid analysis.

∥μmol of residue/μmol of core protein, calculated using a molecular weight of 45,000 for core protein.

¶Determined by chondroitinase ABC digestion, chondroitinase AC digestion, and thiobarbituric acid analysis.

‖Determined from uronate analyses before and after periodate-alkali degradation.

§§Determined from uronate analyses before and after periodate-alkali degradation.

Table II

The proteoglycan isolated by the procedure described so far appeared as a single polydisperse species on gel chromatography, in sedimentation velocity studies, and on SDS-PAGE at low ionic strength in 0.01 M phosphate on 4% disc gels. It appeared to contain a single core protein of M₀, 45,000. Taken together, the studies described above indicated that the proteoglycan isolated following chromatography on Sepharose CL-4B in 4 M GdmCl was a single dermatan sulfate-containing species.

However, further studies involving analytical SDS-PAGE on 4 to 20% gradient slab gels in 0.375 M Tris showed that this was not the case. What appears to be a single, polydisperse species on gel chromatography, sedimentation velocity, and SDS-PAGE at low ionic strength in 0.01 M phosphate, was consistently separated into two species of distinctly different mobilities on SDS-PAGE at high ionic strength in 0.375 M Tris on 4 to 20% gradient slab gels. The appearance of the dermatan sulfate proteoglycan on SDS-PAGE at high ionic strength in 0.375 M Tris on 4 to 20% gradient slab gels is shown in Fig. 8, lane 2. On the gradient slab gel in 0.375 M Tris, the dermatan sulfate proteoglycan is separated into two species. The M₀ of the smaller species, which we call DS-PGII, ranges from 87,000 to 120,000 on the gradient gel. Thus, M₀ of DS-PGII is approximately the same at low ionic strength on disc gels and at high ionic strength on gradient gels, indicating that DS-PGII does not self-associate in 0.375 M Tris. However, the molecular weight of the larger species, which we call DS-PGI, ranges from 165,000 to 285,000 in 0.375 M Tris on the gradient gel. DS-PGI self-associates at high ionic strength at 0.375 M Tris, while DS-PGII does not. This phenomenon results in a clear-cut separation of DS-PGI and DS-PGII contained dermatan sulfate, as demonstrated both by the chondroitinase analyses and by uronate analyses before and after periodate-alkali degradation (Table II). The total glycosaminoglycan in DS-PGI was 45.5% dermatan sulfate and 54.5% chondroitin sulfate, based on chondroitinase analyses. Of the total glycosaminoglycan in DS-PGII, 48.4% was dermatan sulfate and 51.6% was chondroitin sulfate. DS-PGI and DS-PGII contained 34 and 43% dermatan sulfate, respectively. Based on uronate analyses before and after periodate-alkali degradation.

The difference between the values
obtained by periodate degradation and the chondroitinase analyses is probably a reflection of the presence of sulfated iduronic acid residues.

**Immunological Identities of DS-PGI and DS-PGII**—The interactions of the individual species DS-PGI and DS-PGII isolated by preparative electrophoresis with a variety of antisera was studied by ELISA with the results shown in Table III. Antiserum S27 to cartilage-specific proteoglycan monomer from mature bovine articular cartilage reacted strongly with DS-PGI or DS-PGII. This result indicates that DS-PGI and DS-PGII possess different core proteins from cartilage-specific proteoglycan monomer.

Antiserum R166 raised against the mixture of DS-PGI and DS-PGII isolated following chromatography on Sepharose CL-4B in 4 M GdmCl reacted strongly against DS-PGII, but only weakly with DS-PGI. The monoclonal antibody raised against the mixture of DS-PGI and DS-PGII isolated following chromatography on Sepharose CL-4B in 4 M GdmCl was identified as IgG2. It also reacted strongly against DS-PGII, but not at all against DS-PGI. These results suggest that DS-PGI and DS-PGII possess different core proteins and represent two different species of dermatan sulfate proteoglycans.

There was another possible explanation for the limited reactivity of antibodies to DS-PGII with DS-PGI. Studies in progress involving gel chromatography in associative solvents indicate that DS-PGI readily self-associates, while DS-PGII does not. The self-association of dermatan sulfate proteoglycans is mediated via interactions between glycosaminoglycan chains (65-67). Interactions between glycosaminoglycan chains which occur during the self-association of DS-PGI may shield the core protein and interfere with the interaction of antibody with the core protein of DS-PGI.

To examine this possibility, the glycosaminoglycan chains were removed from DS-PGI and DS-PGII with chondroitinase ABC, and the immunoreactivities of their individual core proteins with the monoclonal antibody to DS-PGII were examined by immunoblotting. The individual species, DS-PGI and DS-PGII, separated by preparative electrophoresis were digested with chondroitinase ABC, subjected to SDS-PAGE, and stained with Coomassie Blue. DS-PGI yielded a core protein of Mr, 44,000. DS-PGII showed two Coomassie Blue-stained bands with Mr, 47,000 and 44,000. Immunoblotting was then carried out and the core proteins were reacted with the monoclonal antibody to DS-PGII.

In Fig. 10, lanes A, B, C, and D show the reactions of species with the monoclonal antibody, and lanes A', B', C', and D' show the reactions of the same species with nonimmune ascitic fluid. As shown in lanes A', B', C', and D', some artifactual nonimmune staining is observed at 67,000. As shown in lane C, DS-PGII in its native form reacts strongly with the antibody and appears as a species with Mr, between 77,000 and 98,000 and between 43,000 and 51,000. A trace of material of Mr, 280,000 is also observed. After digestion of DS-PGII with chondroitinase ABC (lane D), major species are observed with Mr, of approximately 43,000 and 47,000.

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**TABLE III**

*Immunoreactivities of the individual species, DS-PGI and DS-PGII, isolated by preparative electrophoresis in 0.375 M Tris*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immune reacting antigen</th>
<th>Antibody binding (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S27 F(ab')2 (1.8 ng/ml)</td>
<td>AID1 from mature bovine articular cartilage</td>
<td>NDb</td>
</tr>
<tr>
<td>NSS4 F(ab')2 (1.8 ng/ml)</td>
<td>Nonimmune sheep serum</td>
<td>NDb</td>
</tr>
<tr>
<td>R166 serum (1:16)</td>
<td>Mixture of DS-PGI and DS-PGII from Sepharose CL-4B</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>R166 PI' serum (1:16)</td>
<td>Preimmune rabbit serum</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Monoclonal antibody from DS ascitic fluid (1:16)</td>
<td>Mixture of DS-PGI and DS-PGII from Sepharose CL-4B</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>Control ascitic fluid (1:16)</td>
<td>Nonimmune</td>
<td>0.15 ± 0.03</td>
</tr>
</tbody>
</table>

* Fraction AID1D1 is from mature bovine articular cartilage.

# Purified proteoglycans were bound at 100 ng (Footnote b) and 50 ng (Footnote c) in 50-μl wells, respectively. Otherwise, details are under "Experimental Procedures." ND, not detectable. Dilutions of sera are indicated. The results show the means of quadruplicate determinations.

NSS, nonimmune sheep serum.

PI, preimmune rabbit serum.
6312

Dermatan Sulfate Proteoglycans

\[ 280 \text{k} \rightarrow 15 \text{k} \]

A B C D

A' B' C' D'

FIG. 10. Immunoblotting of DS-PGI and DS-PGII with and without chondroitinase ABC digestion followed by electrophoresis on a 4 to 20% polyacrylamide gel under reducing conditions. The tracts represent A, DS-PGI native, 40 \( \mu \text{g} \); B, DS-PGI after chondroitinase ABC, 15 \( \mu \text{g} \); C, DS-PGII native, 40 \( \mu \text{g} \); D, DS-PGII after chondroitinase ABC, 15 \( \mu \text{g} \). Molecular weight markers are indicated (\( \times 10^3 \)). Tracks A, B, C, and D were stained with monoclonal antibody to DS-PGI. Tracks A', B', C', and D' were stained with nonimmune ascitic fluid and the variable staining at approximately 67,000 represents artifactual staining.

sizes of these species are strikingly similar to those reported by other workers for chondroitinase-treated dermatan sulfate proteoglycans (68, 69). DS-PGI in its native form (lane A) and after treatment with chondroitinase ABC (lane B) showed absolutely no reaction with the antibody to DS-PGII. These observations suggest that DS-PGI possesses a different core protein and that DS-PGI and DS-PGII represent two different species of dermatan sulfate proteoglycans.

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REFERENCES

Dermatan Sulfate Proteoglycans