Isolation and Characterization of Proteoglycans from the Swarm Rat Chondrosarcoma*

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Proteoglycan monomer (D1) and aggregate (A1) preparations were isolated from 4 M guanidinium chloride extracts of the Swarm rat chondrosarcoma. When EDTA, 6-aminohexanoic acid, and benzamidine were present in the solutions, the D1 preparation contained a single component ($s_0 = 23$ S), and the A1 preparation contained 30% monomer ($s_0 = 23$ S) and 70% aggregate ($s_0 = 111$ S). In the absence of EDTA, 6-aminohexanoic acid, and benzamidine, the A1 preparations contained only small proteoglycan fragments, indicating that extensive enzymatic degradation had occurred.

The composition of the proteoglycan monomer was different from that of proteoglycan monomer preparations from normal hyaline cartilage in that it did not contain keratan sulfate and chondroitin 6-sulfate; only chondroitin 4-sulfate was found.

The A1 preparation from the chondrosarcoma contained only one link protein, which was like the smaller (molecular weight of 40,000) of the two link proteins present in A1 preparations from bovine nasal cartilage. When the A1 preparation from the chondrosarcoma was treated with chondroitinase ABC and trypsin and the digest was chromatographed on Sepharose 2B, a complex was isolated which contained the link protein and the segments of the protein core from the hyaluronic acid-binding region of the proteoglycan molecules.

Proteoglycans are present in the extracellular matrix of normal hyaline cartilage primarily as aggregates (1, 2). Solvents with high concentrations of electrolytes, such as 4 M guanidinium chloride, effectively dissociate the aggregates, minimize noncovalent interactions, and allow the proteoglycan molecules to be extracted from the tissue (3-6). Dialysis to lower electrolyte concentrations allows the molecules to reaggregate, and subsequently density gradient methods can be used to purity both aggregate and monomer proteoglycan preparations (7-9). The chemical and physical characteristics of such preparations isolated from a variety of cartilages have been described (8-21). The monomer preparation from bovine nasal cartilage contains a polydisperse population of macromolecules with molecular weights ranging from less than $1 \times 10^6$ to greater than $4 \times 10^6$. The average molecule contains about 100 chondroitin sulfate chains with average molecular weights of about $2 \times 10^4$ and about 50 keratan sulfate chains with molecular weights of $4 \times 10^4$ to $6 \times 10^4$ (10, 11, 22-24). The polysaccharide chains are attached to a core protein which has an average molecular weight of about $2 \times 10^4$ (25). Recent investigations in a number of laboratories have advanced our understanding of the molecular interactions involved in proteoglycan aggregation (26-31). Those proteoglycans which aggregate interact specifically with hyaluronic acid through a portion of their core protein (26, 28, 29, 32). A number of proteoglycan molecules can bind to a single strand of hyaluronic acid. Small molecular weight link proteins are required to stabilize such aggregate structures (8, 27, 29, 31). A model for the structure and aggregation of proteoglycans from cartilages that is consistent with the available data has been summarized elsewhere (33). This report describes attempts to use the methodology developed with normal cartilages to isolate and purify proteoglycan fractions from a neoplastic tissue, the Swarm rat chondrosarcoma, a tumor first described by Choi et al. (34). A preliminary report of some aspects of this investigation has been presented (35).

EXPERIMENTAL PROCEDURE

Materials—Ultrapure guanidinium chloride (GnHCl), 1 cesium chloride (biological grade), and nonenzymatic protein molecular weight markers were purchased from Schwarz/Mann, 6-aminohexanoic acid and benzamidine hydrochloride hydrate from Aldrich Chemical Co., Sepharose 2B and Sepharose 4B from Sigma, chondroitinase ABC (Proteus vulgaris), 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-β-D-galactose, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfogalactose, and 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-6-O-sulfogalactose standards from Miles Laboratories, trypsin from Miles-Sigma, ovotransferrin from Worthington Biochemical Corp., titanos from Pierce Chemical Co., and UV-225 and Supelcoport (100/120 mesh) from Supelco, Inc. Standard preparations of mucopolysaccharides, including hyaluronic acid with an average molecular weight of 120,000, were gifts from Dr. Martin Mathews, University of Chicago, Chicago, Illinois.

Analytical Procedures—Samples of proteoglycans were dialyzed

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1 The abbreviations used are: GnHCl, guanidinium chloride; Na dodecyl-SO$_4$, sodium dodecyl sulfate. The nomenclature A1, A1-D1, etc. define the proteoglycan fractions as described by Heinegård (9).
against 0.1 M potassium acetate and then water before lyophilization. The amino acid compositions of samples were determined by the procedure of Spackman et al. (90), except that titurionic acid was substituted for stannous chloride as the reductant in the ninhydrin reagent. Samples were hydrolyzed at 110°C in 6 N HCl for 24 to 96 hours in evacuated, nitrogen-flushed tubes. The concentrations of hexosamines in the samples were determined after hydrolysis at 100°C in 4 N HCl for 16 hours as previously described (25).

Protein was established by the method of Lowry et al. (37). Hexuronic acid was determined by the procedure of Bitter and Muir (38). Automated procedures were used for the determination of protein and hexuronic acid in the effluents from columns as described by Hews and coworkers (39). Sialic acid was determined according to the procedure of Joussain et al. (40).

Neutral sugars were determined as glycolyl acetate derivatives by gas-liquid chromatography (41). Samples (0.25 to 3 mg) were hydrolyzed for 4 to 48 hours at 100°C in sealed tubes with 1 ml of Dowex 50-X2 (hydrogen ion form (200 to 400 mesh)) in 0.1 M HCl (40% w/v). After hydrolysis, β-methylglucoside (22 μg) was added to serve as an internal standard. The samples were washed through small (0.2 ml) Dowex 1-X8 xeroborohydride in 1 M NH₄OH for 1 hour at 23°C. The presence of NH₄OH prevents the reduction of the glucuronolactone. The reaction was stopped with 50 μl of glacial acetic acid. The samples were then dried three times in the presence of methanol/HCl (1000/1 v/v), acetylated with 300 μl of acetic anhydride and pyridine at 75°C for 1 hour, and then transferred to tubes (6 × 50 mm) and dried. The residues were dissolved in 5 μl of pyridine, and 0.5-μl aliquots were analyzed. The derivatized sugars were separated with a Hewlett-Packard model 420 gas-liquid chromatograph equipped with a flame ionization detector. The column (2 m × 2 mm) was packed with 3% OV 225 on Supelcoport. The column was run under isothermal conditions at 294°C, detector temperature, 250°C, detector, 250°C, N₂, 15 ml/min, H₂, 15 ml/min, air, 150 ml/min. The oven temperature and injection times and peak heights were used to determine concentrations.

The sulfate contents of samples were determined by the barium chloranilate method of Spencer (42) after prior hydrolysis of the samples in 6 N HCl at 104°C for 20 hours. The relative contents of unsulfated and of the 4- or 6-sulfated disaccharides were estimated by the procedure of Saito et al. (43). Muco polysaccharides in pancreatic digests were identified by electrophoresis on cellulose acetate in 0.05 M sodium acetate, pH 7.0 (9, 29). Flow rates were 3.5 to 4.0 ml/hour, and fractions of 0.5 ml were collected over 4 hours. The protein content of the effluents was measured by the method of Lowry et al. (37).

Preparation of Proteoglycan from Bovine Nasal Cartilage—Cartilage from newborn nasal septa was extracted as described previously (8), except that the solvents for extraction and dialysis contained 0.1 M 6-aminohexanoic acid and 0.01 M NaEDTA (Solution B above). Association and dissociative-dissociative gradients were prepared as described above.

Preparations of Mixed Aggregate—The A1 D1 fraction and the mixture of A1-D2 through A1-D4 were prepared from both the chondrosarcoma and bovine nasal septa. The A1-D1 preparation contained all the nasal chondrosarcoma A1-D1 preparations were dissolved in 4 M GnHCl, 0.05 M sodium acetate, pH 5.8, to the same glucuronic acid concentration as that of the chondrosarcoma A1-D1 fraction. The mixture of A1-D2 through A1-D4 from the nasal cartilage was dialyzed in proportion. Four combinations of the above fractions were prepared: (a) 2 parts of A1-D1 from the chondrosarcoma with 3 parts of the mixture of A1-D2 through A1-D4 from the same tissue; (b) 2 parts of A1-D1 from the chondrosarcoma with 5 parts of the mixture of A1-D2 through A1-D4 from the nasal cartilage; (c) 2 parts of A1-D1 from the nasal cartilage with 3 parts of the mixture of A1-D2 through A1-D4 from the same tissue; and (d) 2 parts of A1-D1 from the nasal cartilage with 3 parts of the mixture of A1-D2 through A1-D4 from the chondrosarcoma.

The mixtures were dialyzed against 0.5 M GnHCl, 0.05 M sodium acetate, pH 5.8, prior to ultracentrifugal analyses.

Interaction of Chondrosarcoma D1 with Hyaluronidase—Chondrosarcoma D1 preparations was dissolved in 4 M GnHCl, 0.05 M sodium acetate, pH 5.8, at a concentration of 4 mg/ml of solution. Identical 0.75-ml aliquots of the solution were added to 0.25-ml aliquots of solutions of hyaluronic acid in H₂O. The mixtures, with up to 25 μg of hyaluronidase, were dialyzed at 4°C against 0.5 M sodium acetate, pH 7.0. The retentates were chromatographed on columns of Sepharose 2B, and effluent fractions were analyzed for hexuronic acid contents. The material that appeared in the excluded volume was taken as measure of the amount of material which interacts with hyaluronic acid (28). A nonaggregating reference proteoglycan was prepared by reducing and alkylating a sample of D1 as described for the proteoglycans of bovine nasal cartilage (3).

Chondroitinase-Aspyn Digests of Chondrosarcoma A1—A chondrosarcoma A1 preparation, 8.25 mg in 1.5 ml, was dialyzed into a buffer (0.1 M Tris-0.1 M sodium acetate adjusted to pH 7.3 with acetic acid), and treated at 37°C for 10 hours with 0.007 M chondroitinase ABC. Under these conditions, 70 to 80% of the susceptible bonds in the chondroitin sulfate chains were cleaved (31). Trypsin (100 μg/ml of 0.001 M HCl) was then added, and the solution was incubated for an additional 4 hours at 37°C. The incubation mixture was chromatographed on a Sepharose 2B column. Effluent fractions were analyzed for protein and hexuronic acid.

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Ultracentrifugal Analyses—Samples for analysis were dialyzed exhaustively against 0.5 M guanidine HCl, 0.05 M sodium acetate, pH 5.8. The final dialyze was used to prepare dilutions by weight. Samples of various proteoglycan preparations were centrifuged at 40,000 rpm, and samples of the proteoglycan core were centrifuged at 52,000 rpm. The analyses were run at 20° in either 12-mm or 30-mm double-sector cells equipped with sapphire windows. Interference patterns were photographed with the camera lens focused on the midplane of the cell and scanned with a Nikon comparator. Integral G(s) curves were constructed from the data as described previously (10) except that the G(s) curves at zero concentration were obtained by extrapolations of ln s values against solute concentrations to zero solute concentration as recommended by Rosenberg et al. (14). For aggregate preparations, the apparent proportions of monomer and aggregate were calculated from the G(s) curves as indicated in Figs. 3, 5, and 6 below. These values were extrapolated directly to zero solute concentration by the use of first order, least squares lines.

Histological Preparation—Small pieces of freshly isolated chondrosarcoma were fixed at 4° with 2.5% glutaraldehyde in 0.05 M sodium phosphate, pH 7.3, for 2 hours. The samples were rinsed at 4° with 4% sucrose in the phosphate buffer and postfixed with 1% osmium tetroxide in the same buffer for 30 min. They were then dehydrated in graded solutions of ethanol and embedded in epoxy resin. Sections (1 μm in thickness) were prepared and stained with 1% toluidine blue in 0.05 M sodium phosphate, pH 9.3.

RESULTS

Characteristics of Swarm Rat Chondrosarcoma—The chondrosarcoma is a composite of nodules of cartilage in a vascular network, Fig. 1. The chondrocytes are pleomorphic. If the tumor is allowed to grow beyond 10 to 15 g, necrotic areas develop. Only tissue which was free of necrosis was used in the experiments described herein.

The tumors lose 93% of their weight when dried at 100°, and proteoglycans, estimated on the basis of hexuronic acid content, account for about 27% of the dry weight. These values are similar to those reported by Choi et al. (34).

Extraction and Purification of Proteoglycans from Chondrosarcoma—Initially, experiments were done to determine how rapidly proteoglycans can be solubilized from the chondrosarcoma in dissociative solvents. When minced tissue was extracted with 5 volumes of solvents containing 4 M guanidine HCl, 95% of the total hexuronic acid was solubilized in 5 hours. Therefore, these were the basic conditions for the extraction step in subsequent experiments. The proportion of the extracting solvent to tissue was lower than that used for extracting normal cartilages because of the higher water content of the tumor.

The chondrosarcoma contained proteases capable of degrading proteoglycans. For example, extracts prepared with Solution A and dialyzed to associative conditions, 0.05 M guanidine HCl, dissolved fibrin clots when incubated at 37°, pH 5.8 to 7.5. Whether these proteases were derived directly from the tumor, from the vascular network around the tumor nodules, or from both has not been determined. Further, aqueous extracts of the tumor degraded proteoglycans at pH 4, in a manner analogous to that described for cathepsins isolated from cartilages (46-50).

The presence of proteases in the extracts impaired the recovery of intact proteoglycan preparations. Therefore, the effects of several protease inhibitors were determined. A1 preparations were isolated with the use of Solution A, Solution B, or Solution C as described in “Experimental Procedure.” A tracing of a schlieren pattern observed in the ultracentrifuge for the A1 preparation isolated with no inhibitors present (Solution A) is shown in Fig. 2a. The solute concentration was about 2 mg/ml. No aggregate component was observed, but several small proteoglycan fragments were found with sedimentation coefficients of 3.5, 7, 10.4, and 12.3 S.

The inclusion of 6-aminohexanoic acid and EDTA (Solution B) in the isolation and purification steps yielded an A1 preparation with significant amounts of aggregate. Integral G(s) curves (51) were constructed from ultracentrifugal analyses of the sample for solute concentrations between 2 and 0.5 mg/ml, Fig. 3. The midpoints of both the monomer and aggregate components were extrapolated to zero concentration to yield weight average sedimentation coefficients, s_w of 23.2 and 78 S, respectively, Fig. 3. The apparent proportions of monomer and aggregate for each concentration were extrapolated to zero concentration in order to correct for the Johnston-Ogston effect (52). The extrapolated values indicated that almost 50% of the weight of the proteoglycan in this preparation was present as aggregate. When an aliquot of this A1 preparation was chromatographed on Sepharose 2B, 53% of the sample was recovered in the excluded volume, Fig. 4b.

The monomer component of the A1 preparation isolated with Solution B showed a bimodal distribution in the ultracentrifuge. This was especially apparent at high solute concentrations, as shown by the inflections in the G(s) curves for C_1 and C_2 of Fig. 3. The data presented in Fig. 5 suggest that this bimodality may be the result of proteolysis. Integral G(s) curves were constructed from the ultracentrifugal data on the monomer, D1, preparation isolated from the same tissue.
FIG. 2. Representative tracings of schlieren patterns observed in the analytical centrifuge are shown. a, chondrosarcoma Al from Solution A, 4,940 s (corrected for acceleration time); b, chondrosarcoma D1 from Solution C, 2,300 s; c, chondrosarcoma Al from Solution C, 1,860 s; d, chondrosarcoma core from chondroitinase digestion of D1, 3,260 s; e, reaggregation of bovine nasal cartilage Al-D1 with chondrosarcoma Al-D2 through Al-D4, 1,220 s; f, reaggregation of chondrosarcoma Al-D1 with bovine nasal cartilage Al-D2 through Al-D4, 1,760 s. The solute concentrations were about 2 mg/ml; the 30-mm centerpiece and a rotor speed of 40,000 rpm were used except for d, where the solute concentration was about 5 mg/ml; the 12-mm centerpiece and a rotor speed of 52,000 were used.

FIG. 3. Integral G(s) curves are presented for four solute concentrations of chondrosarcoma Al from Solution B, 1.83, 1.40, 0.84, and 0.46 mg/ml, for C1 through C4, respectively. The sedimentation coefficients of the midpoints for the monomer and aggregate peaks (●) and their extrapolated s0 values (○) are indicated. The apparent relative proportions of the monomer and aggregate are indicated by the horizontal lines; the extrapolated value of 51% indicates the estimate for the actual proportion of monomer.

FIG. 4. The curves show the hexuronic acid elution profiles from Sepharose 2B for a, bovine nasal cartilage Al; b, chondrosarcoma Al from Solution B; and c, chondrosarcoma Al from Solution C. The percentages indicate the relative areas under the excluded peaks (hatched portions of the profile) to the total.

FIG. 5. Integral G(s) curves are presented for four solute concentrations of chondrosarcoma D1 from Solution C, 2.6, 2.0, 1.3, and 0.65 mg/ml for C1 through C4, respectively. ●, G(s) curve obtained when extrapolations were made at G(s) values of 0.1, 0.2, . . . 0.9 from curves C1 through C4. ○, G(s) curve obtained from the similar extrapolation for the monomer component in the chondrosarcoma Al curves shown in Fig. 3. The A1 preparation (see Fig. 3) is indicated by the dashed line in Fig. 5. The latter curve has a pronounced skewed distribution toward lower sedimentation coefficients, indicating that the monomer in this A1 preparation contained a larger fraction of molecules with lower sedimentation coefficients than for the molecules in the D1 preparation.

When benzamidine, an inhibitor of trypsin-like activity (53), was used in addition to EDTA and 6-aminohexanoic acid, Solution C, proteolysis was inhibited more effectively. The

The D1 fractions isolated with Solutions A and B had the same centrifugal characteristics.
G(s) curves, Fig. 6, and the chromatographic analysis, Fig. 4c, indicate that about 70% of the proteoglycan molecules in this A1 preparation were present as aggregates. The value of $s_1$ for the aggregate component increased to 111 S. The G(s) curves for the monomer component yielded an extrapolated curve at zero concentration identical with that for the D1 preparation shown in Fig. 5. A tracing of the schlieren pattern for the A1 preparation isolated with Solution C is shown in Fig. 9.

Centrifugal and Chromatographic Analyses of Bovine Nasal Cartilage A1—For comparison, experiments were done with bovine nasal cartilage. The A1 preparation was isolated with the use of Solution B. Integral G(s) curves, Fig. 7, indicated that the monomer and aggregate components in this preparation had extrapolated weight average sedimentation coefficients of 27.7 and 126 S, respectively. The centrifugal analyses also suggested that the aggregate fraction accounted for about 85% of the sample. The Sepharose 2B chromatogram, Fig. 4a, showed that about 84% of the sample was excluded.

Composition of Chondrosarcoma D1—The chemical composition of the chondrosarcoma D1 fraction is summarized in Table I. This preparation accounted for 90% of the hexuronic acid originally present in the tissue. The amino acid composition was similar to that reported for monomer proteoglycans isolated from several cartilages (9, 10, 12, 14, 17).

Protein accounted for about 7% of the dry weight of the preparation. Glucuronic acid, galactosamine, and sulfate were present in approximately equimolar amounts, characteristic of chondroitin sulfate. Calculations based on these values indicate that the potassium salt of this polysaccharide accounted for 90 to 93% of the dry weight of the sample. Electrophoresis of papain digests on cellulose acetate showed only one band, which migrated to the same position as that found for standard chondroitin 4-sulfate. Paper chromatography was used to separate the unsaturated disaccharides generated by digestion of the D1 preparation with chondroitinase, Table II. Only 4-sulfated (87%) and nonsulfated (13%) disaccharides were detected. The glucuronic acid to xylose ratio was slightly greater than 40, suggesting that the chondroitin sulfate chains have an average length of 40 disaccharide repeat units. The galactose to xylose ratio of about 2:1 suggests that the normal linkage region oligosaccharide (galactosyl-galactosyl-xylosyl-serine) between the chondroitin sulfate chains and the protein core (55), is present. Neutral sugars that are typical for skeletal keratan sulfate (23, 24, 56–59) were not excluded.

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**Table I**

| Amino Acid       | umol/g dry weight | Amino acid residues/1000 residues |
|------------------|-------------------|----------------------------------|
| Lysine           | 10.0              | 17.6                             |
| Histidine        | 13.4              | 23.5                             |
| Arginine         | 16.6              | 29.2                             |
| Aspartic acid    | 41.4              | 22.6                             |
| Threonine        | 53.1              | 93.1                             |
| Serine           | 76.7              | 134.5                            |
| Glutamic acid    | 75.6              | 132.6                            |
| Proline          | 47.1              | 82.7                             |
| Glycine          | 28.9              | 158.4                            |
| Alanine          | 36.4              | 63.7                             |
| Half-cystine     | 4.3               | 7.4                              |
| Valine           | 31.0              | 54.0                             |
| Methionine       | 3.0               | 5.2                              |
| Isoleucine       | 16.1              | 28.2                             |
| Leucine          | 42.4              | 74.3                             |
| Tyrosine         | 8.8               | 15.6                             |
| Phenylalanine    | 15.6              | 27.3                             |
| Protein (Lowry method) | 67.5 mg |                       |
| Sum of amino acid residues | 69.1 mg |                       |
| Glucosamine      | 22.8              |                                  |
| Glucosamine      | 98.8*             |                                  |
| Galactosamine    | 1700              |                                  |
| Alkali-labile galactosamine | 33.4* |                  |
| Sialic acid      | 28.0*             |                                  |
| Glucuronic acid  | 1748              |                                  |
| Sulfate          | 9147              |                                  |
| Galactose        | 89.3*             |                                  |
| Xylose           | 42.5*             |                                  |
| Mannose          | <0.1*             |                                  |
| Fucose           | <0.1*             |                                  |

* Dried for 8 hours at 78° over P₂O₅ in vacuo.
* Calculated from the values for a chondroitinase ABC core preparation purified on Sepharose 4B and corrected on the basis of the Lowry protein value.
*· The amount of galactosamine destroyed by treating a core sample with 1 M NaBH₄ in 0.05 M NaOH at 4° for 64 hours (64).
* Maximal values obtained during the time course of acid hydrolysis over 44 hours.
found. However, the purified core preparations isolated from chondroitinase-digested chondrosarcoma D1 did contain approximately equimolar amounts of glucosamine, sialic acid, and alkali-labile galactosamine (54), Table I. These carbohydrate residues are characteristically found in skeletal keratan sulfate (23, 24, 56–59). These results suggest that the chondrosarcoma D1 preparations may contain the initiation oligosaccharides for the keratan sulfate chains.

Characteristics of Chondroitinase-digested Chondrosarcoma D1—The D1 core preparation showed a single peak in the ultracentrifuge, Fig. 2d, with a s, of 7.3 S. A symmetrical, included peak was eluted from Sepharose 2B and Sepharose 4B with K, values of 0.74 and 0.60, respectively. These values are similar to those observed in comparable experiments with preparations from the proteoglycan monomer of bovine nasal septa (25, 29, 30).

Interaction of Chondrosarcoma Proteoglycan with Hyaluronic Acid—One of the requisites for proteoglycan aggregation is the ability of proteoglycan monomers to interact with hyaluronic acid (26–31, 60). Chromatography of proteoglycans with and without hyaluronic acid on Sepharose 2B can be used to monitor this interaction (28). The elution profile for the chondrosarcoma D1 without added hyaluronic acid is shown in Fig. 8a; an included peak (K = 0.30) was found. The addition of 12 µg of hyaluronic acid to an identical D1 sample resulted in a shift of material into the excluded volume of the column, Fig. 8b. The elution profiles of D1 admixed with different concentrations of hyaluronic acid showed that the percentage of D1 molecules excluded increased with hyaluronic acid concentration up to 20 µg of hyaluronic acid/3 mg of D1, at which ratio 55% of the D1 molecules were excluded. Reduction and alkylation of the D1 preparation completely destroyed the ability of the proteoglycans to bind to hyaluronic acid. Previously it was shown that such treatment also prevents aggregation of bovine nasal proteoglycan preparations (3, 8).

Properties of Aggregate from Rat Chondrosarcoma—At least one of the two small molecular weight link proteins (61) present in A1-D4 preparations from bovine nasal cartilage is necessary for aggregation (27, 29, 31). Therefore, the A1-D4 fraction was isolated from the chondrosarcoma A1 and compared with the same fraction isolated from bovine nasal cartilage. Samples were subjected to electrophoresis on 7% polyacrylamide gels in the presence of Na dodecyl-SO₄, A1-D4 from bovine nasal cartilage showed two predominant protein-staining bands, Fig. 9b, as previously observed (29, 60). Their molecular weights were about 45,000 and 40,000, as estimated from graphs of logarithm of molecular weight against mobility of proteins with known molecular weights on identical gels. In contrast, the chondrosarcoma A1-D4 fraction contained only a single major protein component, with an estimated molecular weight of 40,000, Fig. 9a. When the two A1-D4 preparations were mixed, the protein in the chondrosarcoma A1-D4 underwent co-electrophoresis with the smaller of the two proteins in the nasal cartilage A1-D4, Fig. 9c.

Reaggregation Experiments—Because of the difference in the A1-D4 fractions, attempts were made to determine if they could be reinterchanged in reaggregation experiments. Mixtures of the A1-D1 fractions with the A1-D2 through A1-D4 fractions were prepared and treated as described under “Experimental Procedure.” The A1-D2 and A1-D3 fractions were included because it has been shown that these fractions contain the hyaluronic acid molecules required for the formation of aggregates with normal cartilages (28, 60). Fig. 2e shows a tracing of a schlieren pattern for a mixture in which chondrosarcoma A1-D2 through A1-D4 was added to bovine nasal A1-D1, while Fig. 2f shows a tracing of the converse experiment, i.e. a mixture of bovine nasal A1-D2 through A1-D4 with chondrosarcoma A1-D1. The mixture which contained only chondrosarcoma fractions gave a pattern identical with that in Fig. 2e, while the mixture which contained only bovine nasal fractions gave a pattern identical with that in Fig. 2f. Both the chondrosarcoma and bovine nasal A1-D2 through A1-D4 fractions promote aggregation with heterologous as well as homologous proteoglycan preparations. However, in mixtures that contained the chondrosarcoma A1-D2 through A1-D4, a somewhat smaller amount of aggregate was observed.

Chondroitinase-Trypsin Digestion of Chondrosarcoma A1—A brief treatment of bovine nasal A1 with chondroitinase followed by digestion with trypsin yielded a large molecular weight complex that contained hyaluronic acid, the smaller link protein, and a large segment of protein, referred to as the hyaluronic acid binding region, from the proteoglycan molecules (31). The chondrosarcoma A1 fraction was treated in a similar manner. Chromatography of the chondroitinase ABC-trypsin incubation mixture on Sepharose 2B gave the pattern shown in Fig. 10a. The protein-hyaluronic acid complex eluted as a broad peak shortly after the excluded volume; it was isolated and subjected to electrophoresis on a Na dodecyl-SO₄ polyacrylamide gel, Fig. 10b. A protein band was seen in the same position as the 40,000 molecular weight protein from the chondrosarcoma A1-D4 fraction, Fig. 9a. Two additional, closely spaced protein bands were observed at positions in the gel where proteins with molecular weights of about 55,000

### Table II

| Standard unsaturated disaccharide | Rₐ* | Rₐ unknown | Percentage of total |
|---------------------------------|-----|-------------|---------------------|
| Non-sulfated                    | 0.43 | 0.43        | 13                  |
| 4-Sulfated                      | 0.30 | 0.30        | 87                  |
| 6-Sulfated                      | 0.17 |             |                     |

* Rₐ in 1-butanol/acetic acid/ΝΗ₄OH, 2/3/1, v/v/v, 17 hours, 23°.

* Determined as described by Saito et al. (43).
FIG. 9. (left). Photographs of Na dodecyl-SO₄ polyacrylamide gels and tracings of densitometric analyses of the gels are shown for a, chondrosarcoma A1-D4, b, bovine nasal cartilage A1-D4, and c, 1:1 mixture of chondrosarcoma and bovine nasal cartilage A1-D4. Approximately 20 μg of protein were used for each analysis.

FIG. 10 (right). The curves in a show the hexuronic acid (---) and 65,000 would migrate. These proteins correspond to the hyaluronic acid-binding polypeptide fraction derived from the core protein of proteoglycan molecules in the analogous experiment with bovine nasal A1 (31). However, in the latter case only one broad, polydisperse band, with an average molecular weight of approximately 90,000 was observed; the polydispersity was attributed to the presence of some keratan sulfate in the preparation (31). The fact that two closely spaced, narrow bands were observed in the experiment with the chondrosarcoma A1, which does not contain keratan sulfate, suggests that there may be two different, though related polypeptides in this fraction. Alternatively, a peptide bond in the hyaluronic acid-binding region of the proteoglycan molecules may be partially resistant to treatment with trypsin, and limit digestion may not have been reached. It is of interest, however, that two proteins were present in small concentrations in the chondrosarcoma A1-D4 preparation which had mobilities similar to those for the two closely spaced bands in the chondroitinase-trypsin experiment, Fig. 9b. This is consistent with the suggestion by Heinegård and Hascall (33, 62) that proteoglycans in A1 preparations which are substituted with few or no polysaccharide chains contain little or no polypeptide beyond the hyaluronic acid-binding region polypeptide (see also the discussion by Rosenberg (63)). The ratio of the area under the link protein peak to that under the two hyaluronic acid-binding region polypeptides, Fig. 10b, was about 1.3. If the amounts of dye bound per unit weight of protein were equivalent, these results would suggest that the original hyaluronic acid-protein complex contained two link proteins for each hyaluronic acid-binding region polypeptide. In contrast, it was suggested that there was only one link protein for each hyaluronic acid-binding region protein in A1 preparations from bovine nasal cartilage (29).

DISCUSSION

In general, the data in this paper indicate that the chondrosarcoma contains proteoglycan aggregates with structures consistent with the emerging model for aggregates from normal cartilages (33). The A1 preparation obtained when EDTA, 6-aminohexanoic acid, and benzamidine were used in the isolation steps represented about 90% of the total tissue hexuronic acid. It had properties very similar to those reported for bovine nasal and tracheal A1 preparations (29) with respect to the following parameters: (a) monomer and aggregate size; (b) the presence of a large proportion of aggregate; (c) the ability of the monomer preparation to interact with hyaluronic acid; and (d) the presence of a hyaluronic acid-protein complex which resists digestion with chondroitinase and trypsin. The chondrosarcoma A1 differs from A1 preparations from

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*For an equimolar complex the ratio of the areas of the peaks should be proportional to the ratio of the molecular weights, which in this case is 4 x 10⁵:6 x 10⁴ or 0.67. Since the actual ratio is 1.3, a complex of two link proteins and one hyaluronic acid-binding region polypeptide of the proteoglycan monomer (D1) is suggested.*
normal hyaline cartilages with respect to the following parameters: (a) the lack of keratan sulfate in the proteoglycan molecules; (b) the absence of chondroitin 6-sulfate; and (c) the presence of only one of the two link proteins. Further, two polypeptides were derived from the hyaluronic acid-binding region of the proteoglycans when chondrosarcoma A1 was treated with chondroitinase and trypsin, while only one was observed in the comparable experiment with bovine nasal cartilage A1 (31). The presence of keratan sulfate in the latter preparation, however, may introduce enough polydispersity such that two populations of polypeptides, if present, would not be observed. The absence of keratan sulfate and chondroitin-6-sulfate suggests that the chondrosarcoma proteoglycans are more nearly like those that are present in immature or embryonic cartilages (34, 64).

Choi et al. (34) examined the characteristics of the poly saccharides isolated from the Swarm rat chondrosarcoma after papain digestion. They found hyaluronic acid (1.2%), chondroitin 4-sulfate (98%), and a separate fraction which contained glucosamine but was not keratan sulfate. The presence of glucosamine, sialic acid, and alkali-labile galactosamine in the chondrosarcoma D1 preparation suggests that this latter fraction may well be an oligosaccharide fraction related to that normally involved in the attachment of keratan sulfate chains to the core protein.

The isolation of proteoglycans from the chondrosarcoma by procedures previously applied to hyaline cartilages yielded preparations with proteoglycans that were partially degraded. The neoplastic tissue or its vascular network (or both) contained enzymes, presumably proteases, which retained some activity even after exposure to 4 M GdnHCl. Inclusion of the protease inhibitors 6-aminohexanoic acid, EDTA, and benzamidine hydrochloride in the solutions used for extraction and dialysis, and expeditious manipulation at low temperature, greatly reduced or completely abolished the effects of these endogenous enzymes. Because hyaline cartilages contain small amounts of proteases active at both acidic and neutral pH (46-50), it is advisable to utilize protease inhibitors and low temperatures in preparing the A1 fractions of proteoglycans. For example, the use of 6-aminohexanoic acid and EDTA in the isolation of bovine nasal A1 yielded a preparation in which about 85% of the material was aggregated, with a sedimentation coefficient of 126 S. This value for the sedimentation coefficient is somewhat higher than previously reported for aggregate in A1 preparations from this tissue. 90 to 100 S (8, 29). This preparation also had a higher aggregate content than did the A1 preparations isolated by the two step procedure recommended by Hardingham and Muir (28).

Hardin and Muir described the isolation of a proteoglycan fraction extracted from pig laryngeal cartilage with 0.15 M NaCl at 4°C and at neutral pH which (a) accounted for about 15% of the extractable proteoglycan molecules, (b) was smaller than the bulk of the proteoglycan molecules, (c) would not interact with hyaluronic acid, and (d) contained very high ratios of chondroitin sulfate to protein and chondroitin sulfate to keratan sulfate (28). A similar proteoglycan preparation from bovine nasal cartilage was described by Mayes et al. (17). In the present study low molecular weight proteoglycan molecules were not detected by centrifugal analyses of the chondrosarcoma A1, Fig. 6, and of the bovine nasal A1, Fig. 7. The monomer component of the chondrosarcoma A1 accounted for 30% of the total sample and gave the same extrapolated G(s) curve as that for the D1 monomer, Fig. 5.

The monomer component of the nasal A1 preparation accounted for 15% of the total sample and gave an extrapolated G(s) curve showing slightly higher average sedimentation coefficients than that reported previously for the D1 preparation from bovine nasal cartilage (10). When a chondrosarcoma A1 fraction was prepared in the absence of benzamidine, however, lower molecular weight proteoglycan molecules were detected in the monomer fractions, Figs. 3 and 5. These results suggest the possibility that the proteoglycans recovered in the low salt extracts from normal cartilages (17, 28) may have been partially degraded. The properties of the low salt proteoglycan preparations, described above, would be consistent with this hypothesis. Proteoglycans in aggregates have an asymmetric structure in which keratan sulfate chains are concentrated on the protein core near the hyaluronic acid-binding region of the polypeptide while the chondroitin sulfate chains are concentrated more distally (33). The enzymatic scission of a bond in the middle of the polypeptide would release a readily extractable, smaller proteoglycan fragment that would be enriched in chondroitin sulfate over both protein and keratan sulfate. The rest of the proteoglycan molecule would remain bound in the aggregate structure and would not be extracted in the low salt solvents.

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