Swarm Rat Chondrosarcoma Proteoglycans

PURIFICATION OF AGGREGATES BY ZONAL CENTRIFUGATION OF PREFORMED CESIUM SULFATE GRADIENTS*

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A procedure utilizing zonal centrifugation on preformed cesium sulfate gradients (0.15 to 0.50 M) is described for purifying the aggregate fraction from cartilage proteoglycan samples. The procedure permits rapid (9 h) separation of aggregates from nonaggregated proteoglycans at centrifugation temperatures of 2 to 11 °C with a high loading capacity (up to 100 mg of aggregate in one preparative run). Hexuronic acid analyses are used directly on gradient fractions without interference to monitor proteoglycan distributions. For the proteoglycan fraction obtained from the Swarm rat chondrosarcoma, using an associative extraction procedure and an associative isopycnic density gradient purification step, about 75% of the total hexuronic acid was recovered in aggregates and about 25% in nonaggregated proteoglycans. Aggregating monomers were obtained from purified aggregate by centrifugation in an isopycnic dissociative CsCl density gradient. The nonaggregated proteoglycans were fractionated further on the Cs2SO4 zonal gradients into slower and faster sedimenting populations. Each of the nonaggregated populations as well as the aggregating population was subfractionated on dissociative glycerol zonal gradients into two or three groups according to differences in their average sedimentation rates. Analyses of the subfractions for (a) antigenicity with antibodies directed against either proteoglycan monomer or the purified hyaluronic acid-binding region, (b) average chondroitin sulfate chain sizes, and (c) chemical composition suggest: 1) polydispersity of aggregating monomers is primarily the result of differences in the average chondroitin sulfate chain sizes on different molecules; 2) about 15% of the aggregating monomers contain a discretely smaller core which was observed after chondroitinase digestion (Kimata, K., Hascall, V. C., and Kimura, J. H. (1982) J. Biol. Chem. 257, 3827-3832).

Proteoglycans are present in cartilages primarily as high molecular weight aggregates (1) in which proteoglycan monomers interact specifically with hyaluronic acid and link proteins (2-5). The monomers contain a central core protein having structural and functional polarity. Chondroitin sulfate chains are covalently attached at one end (about 60% of the total), while the other end (20-30%) contains the hyaluronic acid-binding region essential in the formation of the aggregates. Other oligosaccharide chains and usually keratan sulfate chains are also present on the core protein (6-8).

Aggregates are preferentially recovered in the bottom (A1) fraction after equilibrium sedimentation on associative CsCl density gradients (3, 9). However, usually about 25%, but occasionally 50% or more of the proteoglycans in A1 fractions, are not aggregated. Several biochemical and electron microscopic studies (6, 9, 12-17, among others) have shown that proteoglycan monomers derived from A1 fraction are very heterogeneous with an average of 2.5 x 10^4. On the basis of the analyses of lower buoyant density fractions of monomers derived from dissociative density gradients of A1, a model has been proposed suggesting that the population of proteoglycans that aggregate contains a constant hyaluronic acid-binding region and an adjacent keratan sulfate-rich region possibly representing a selective breakdown product of the major population; 3) the nonaggregated proteoglycan fraction of smallest size (about 10% of the original sample) is distinctly different from aggregating monomers; 4) the nonaggregated proteoglycans of larger size (about 15% of the original sample) may be related to aggregating monomers but still have some compositional and antigenic differences; and 5) the extent of polydispersity of the largest nonaggregated proteoglycan fraction is similar to that of the aggregating monomers and is likewise primarily related to differences in the average chondroitin sulfate chain sizes on different molecules.

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1 The abbreviations used for identifying proteoglycan fractions follow the systematic nomenclature of Heinegard (10) where A1 indicates the proteoglycan fraction which contains aggregates, isolated from the bottom of an associative density gradient; D1 is the monomer fraction isolated from the bottom of a dissociative gradient. In addition, aA1 are aggregate fractions isolated from the Swarm rat chondrosarcoma directly from associative extracts without a prior dissociation step (11). Asg and Agg-D1 are aggregates from the bottom of an associative Cs2SO4 zonal gradient and its monomer fraction, respectively; NA-S and NA-P are subfractions of nonaggregated proteoglycans from an associative Cs2SO4 gradient which sediment slower and faster, respectively; a, b, and g are subfractions from a dissociative glycerol gradient which are designated as in Fig. 4.
tion but that the chondroitin sulfate-rich regions of the core are variable in size (6, 15, 18). However, more recently, evidence has been presented suggesting that the aggregating proteoglycan population from most hyaline cartilages contains two discrete subpopulations (19).

Earlier studies using sequential extraction with low and subsequent high ionic strength solvents demonstrated that cartilages also contain proteoglycans which are not aggregated (18, 20). However, the low ionic strength solvents without protease inhibitors may have yielded some degradation products from aggregating proteoglycans. A recent report (21) described the properties of nonaggregated proteoglycans separated from the A1 fraction of the same tissue using associative rate zonal sedimentation in a preformed NaCl density gradient similar to the method described originally by Franek and Dunstone (23).

These studies demonstrate that A1 fractions as currently isolated contain nonaggregated proteoglycans. The first purpose of this paper is to describe a rapid, convenient, and high capacity method for separating and purifying the proteoglycan aggregate population from nonaggregated proteoglycans using zonal centrifugation on a preformed CsSO4 gradient.

The second purpose is to describe the characteristics of aggregating and nonaggregating proteoglycans purified from the A1 fraction obtained from the Swarm rat chondrosarcoma using this method.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ultrapure CsSO4 was purchased from Sigma. Ultrapure guanidine HCl and CsCl were from Schwarz/Mann; Bio-Gel A-1.5m (200–400 mesh) was from Bio-Rad; polyvinyl and polystyrene microtiter plates were from Cook Laboratories; and horseradish peroxidase conjugated to goat anti-rabbit IgG and pig skin hyaluronic acid were from Miles Laboratory. High molecular weight hyaluronic acid from rooster comb was kindly provided by Seikagaku Kogyo, Japan. Molecular weight markers for hyaluronic acid (M, values of 25,400, 19,200, and 12,400 with Kav values on Sepharose 6B of 0.45, 0.51, and 0.59 (24)) were a kind gift from Dr. Å. Wasteson, Uppsala, Sweden.

A proteoglycan fraction, A1, was isolated from the Swarm rat chondrosarcoma by associative extraction and direct associative density gradient centrifugation as described previously (11). Approximately 70% of the proteoglycans in this preparation are aggregated. The monomer, aA1-D1, fraction was prepared from the A1 with dissociative density gradient centrifugation as described previously (11).

The complex of hyaluronic acid-binding region, link protein, and hyaluronic acid was purified after clostripain digestion of aAl as described in detail elsewhere (28). The weight of proteoglycan was calculated by assuming that 25% by weight is hexuronic acid. About 50% of the hexuronic acid was recovered as aggregate (Agg) in the fractions near the bottom cushion as described under "Results." The nonaggregated proteoglycans in the upper fractions were recovered and dialyzed, and high molecular weight hyaluronic acid from rooster comb was then added to give a ratio of proteoglycan to hyaluronic acid of about 50:1. After 3 h at 10 °C, the mixture was applied to CsSO4 density gradients and centrifuged as described above. The hexuronic acid distributing in the upper fractions as nonaggregated proteoglycan was recovered for further study (see Fig. 3n, below).

**Aggregation Monomers from Purified Aggregates**—The aggregate fraction recovered at the bottom of the preparative gradients, see Fig. 3e below, was desalted by concentration on an Amicon PM-10 membrane at 4 °C and dialyzed with 0.2 M Tris-HCl-0.2 m sodium acetate, pH 7.2. An equal volume of 8 m guanidine HCl (Gdn-HCl) was then added, and the solution was kept at 37 °C for 24 h to release glycosaminoglycan chains (31). The solutions were then incubated with phosphate-buffered saline-0.05% Tween 20, pH 7.0. Goat anti-rabbit IgG (25) was dissolved in 300 μl of phosphate-buffered saline-0.05% Tween 20, pH 7.0, at a concentration of 70% ethanol (v/v) and 1% potassium acetate (w/v). The precipitates were collected by centrifugation. Each of the precipitates was dissolved in 47.6 cm 3 of 0.1 M sodium acetate-0.1 M Tris-HCl, pH 7.2, then coated with the above buffer, and

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200 µl of O-phenylenediamine-0.03% H₂O₂ (v/v) were added as substrate. The color was allowed to develop at room temperature for 90 min, and the reaction was stopped by adding 50 µl of 8 M H₂SO₄ to each well. The color was measured spectrophotometrically at 492 nm with a Titertek Multiskan plate reader. The data were analyzed using the method of Rodbard (32).

**Analytical Methods**—Amino acid and hexosamine analyses were done with a Durrum D-500 amino acid analyzer as described previously (25). Hexuronic acid contents were determined by an automated procedure using the carbazole method (33). Sialic acid was measured by an automated procedure (7) using the resorcinol method of Jourdan et al. (34).

**RESULTS**

**Parameters of Centrifugation**—Standard conditions were developed for rate zonal sedimentation of proteoglycan preparations on preformed Cs₂SO₄ linear gradients. The use of Cs₂SO₄ for the gradient provides a satisfactory density stability over a relatively narrow electrolyte concentration range and permits direct analysis for hexuronic acid by both manual and automated procedures without interference, in contrast to other rate zonal sedimentation methods using sucrose (29), glycerol (30), or halide salts (22, 23) for the gradient.

Several parameters were investigated to achieve satisfactory results. With 6-h centrifugation in standard conditions, Fig. 1, bottom, 26% of the hexuronic acid was recovered in nonaggregated proteoglycan fractions (fractions 1-14), and baseline hexuronic acid values separated this peak from the aggregate at the bottom (fractions 20-25). When the gradient was twice as steep, Fig. 1, top, 35% of the hexuronic acid remained in the nonaggregated proteoglycan fractions, and there was incomplete resolution between the nonaggregated proteoglycan peak and the aggregate fraction. Shallower gradients introduced instability and did not have as much capacity as the standard conditions (data not shown).

Different times of centrifugation were tested in which identical samples of aA1 were centrifuged at 25,000 rpm and 11 °C. The amount of hexuronic acid in the slower sedimenting fractions relative to that in the aggregate fractions decreased with time until about 5 h. After that, there was no significant decrease in the proportion of hexuronic acid in the slower sedimenting peak, although the peak position migrated further into the gradient with time (data not shown). The 6-h time point was selected for further experiments, since, as indicated in Fig. 1, bottom, base-line hexuronic acid values separate the two components.

**Fig. 1.** Rate zonal sedimentation on Cs₂SO₄ zonal density gradients of different steepnesses. An aA1 sample with 1.32 mg/ml as hexuronic acid was applied. The gradient and samples were prepared in 0.1 M Tris-HCl-0.1 M sodium acetate, pH 7.2. The centrifugation was done in a Beckman SW 27.1 rotor for 6 h at 11 ± 2 °C.

**Fig. 2.** Effect of temperature and loading solute concentration on the sedimentation. The indicated concentrations of aA1-D1 as hexuronic acid were centrifuged on the Cs₂SO₄ density gradients for 6 h at either 11 ± 2 °C (a to c) or 4 ± 2 °C (d to f). Arrows indicate peaks of hexuronic acid content.

The experiment shown in Fig. 2 tested both the effect of solute concentration and temperature on the sedimentation of aA1-D1 monomer in the zonal gradient under standard conditions. Concentrations of proteoglycan between 0.4 and 2.0 mg/ml as hexuronic acid gave similar profiles. At higher concentrations, the macromolecules sedimented somewhat more slowly, and there was an asymmetrical broadening of the profiles. At the lower temperature, 4 °C, the profiles were essentially the same as at 11 °C except that the peak fractions occurred about one fraction earlier at each concentration. The results indicate that the gradients have a high solute capacity, achieving satisfactory results with up to 8 mg of proteoglycan (2.0 mg of hexuronic acid)/ml in the applied sample, and that the method is relatively insensitive to differences in temperature. Usually, concentrations with 1.0-1.5 mg hexuronic acid/ml were used in the experiments.

The small peak of hexuronic acid in the aggregate fractions at the bottom of the gradient for each profile in Fig. 2 represents proteoglycans bound to the small amount of hyaluronic acid often present in aA1-D1 monomer fractions (see next section).

**Preparation of Aggregate and Aggregate-D1**—Preparative rate zonal centrifugation was used to isolate a large amount of aggregate from an aA1 preparation from the Swarm rat chondrosarcoma. As for the samples described above (Fig. 1, bottom), about 70% of the proteoglycans were recovered in the aggregate fraction. The nonaggregated proteoglycan fraction is described below. Proteoglycan monomers in the aggregate fraction were separated from the other components of the aggregate, namely hyaluronic acid and link protein as well as from some aggregating monomers with lower buoyant densities, by centrifugation in an isopycnic dissociative (4 M guanidine HCl) gradient as described under "Methods." About 90% of the hexuronic acid was recovered in the bottom fraction which will be referred to as Agg-D1. About 7.0, 2.5, and 1.2%
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of the hexuronic acid were recovered in Agg-D2, Agg-D3, and Agg-D4, respectively. Aliquots of the purified aggregate and Agg-D1 were centrifuged on standard analytical zonal gradients, Fig. 3, d and e. The hexuronic acid profile for the aggregate sample was entirely in the bottom fractions, indicating that there was no remaining nonaggregated proteoglycan present and that the aggregate remained stable under the conditions used for its isolation and purification. The majority of Agg-D1 sediments as a broad peak near the middle of the gradient. However, a small proportion of hexuronic acid in the Agg-D1 fraction also sediments to the bottom of the gradient. This is probably due to the presence of small amounts of hyaluronic acid remaining in the Agg-D1 fraction as has been observed for monomer preparations previously (3). Aggregating monomers would saturate this residual hyaluronic acid and the complex would sediment to the bottom. When dissociative solvents were used in the zonal gradients as described in Fig. 4 below, no proteoglycan sedimented into the bottom fractions.

Subfractionation of Nonaggregated Proteoglycans—On the preparative zonal gradients, the majority of the nonaggregated proteoglycans was broadly distributed in the fractions in the upper two-thirds, similar to Fig. 1, bottom. It is possible that the nonaggregated proteoglycan fraction could contain proteoglycans which are able to interact with hyaluronic acid but which were not recovered in the aggregate due to insufficient amount of hyaluronic acid in the aA sample. Such potentially aggregating proteoglycans should be recovered in the bottom fraction of zonal gradients after adding exogenous high molecular weight hyaluronic acid. The nonaggregated proteoglycan fraction was incubated for 3 h at 10°C with 2% hyaluronic acid, and the mixture was applied to the standard analytical zonal gradients and recentrifuged. Less than 5% of the total hexuronic acid was recovered in the aggregate fraction, Fig. 3a, indicating that the proteoglycans in the nonaggregated fraction did not bind to hyaluronic acid to any significant extent. However, since an asymmetrical sedimentation profile was observed, the preparation was subdivided into two fractions: a slower sedimenting fraction corresponding to fractions 1–8 and a faster sedimenting fraction corresponding to fractions 9–16 in Fig. 3a, referred to as NA-S and NA-F, respectively. Recentrifugation of NA-S and NA-F on

Fig. 3. Distribution of hexuronic acid on associative Cs₂SO₄ density gradients. a, NA, nonaggregated proteoglycan, 1.5 mg/ml as hexuronic acid plus hyaluronic acid, 0.12 mg/ml. The mixture was incubated for 3 h at 10°C, then applied to the gradient. Portions of NA-S (b), the slower sedimenting fractions of NA (pooled fractions 1 to 8 from a), and of NA-F (c), faster sedimenting fractions of NA (pooled fractions 9 to 15 from a) were recentrifuged in identical gradients. Aliquots of Agg-D1 (d) and of the aggregate (e) were recentrifuged. All gradients were done under standard conditions for 6 h.
standard analytical gradients gave the reproducible sedimentation profiles, Fig. 3, 6 and c, indicating that the two subfractions clearly differ in their sedimentation rates. A large proportion of the NA-F fraction sedimented in the same region as proteoglycans in Agg-D1, indicating that NA-F contains proteoglycans with a broad range of sizes. The majority of the macromolecules in NA-S had slower sedimentation rates than those in the NA-F and the Agg-D1 fractions.

Size Subfractionation of Proteoglycans in Dissociative Zonal Gradients—Aliquots of aggregate and Agg-D1 were dissociated in 4 M guanidine HCl and centrifuged in dissociative zonal gradients made of 15-35% glycerol in 4 M guanidine HCl-0.02 M Tris-HCl, pH 8.0 (30). Sedimentation rates of proteoglycans on this gradient correspond roughly to their hydrodynamic sizes (29). Both samples gave essentially the same broad sedimentation profile of hexuronic acid as shown in Fig. 4, indicating the polydispersity of aggregating monomers. Subfractions from the aggregate and the Agg-D1 profiles were then isolated as indicated in Fig. 4 and referred to as α, β, and γ, according to their sizes.

Aliquots of NA-S and NA-F were also centrifuged on dissociative zonal gradients in the same way. NA-S gave two peaks of hexuronic acid which were almost equivalent in amount, referred to as α and β as shown in Fig. 4. The NA-S-α was the slowest sedimenting proteoglycan fraction and contained approximately 11% of the total hexuronic acid in the original aA1 fraction. The NA-S-β fraction also sedimented somewhat slower than the aggregating monomers. These results indicate that the hydrodynamic sizes of proteoglycans in NA-S are definitely smaller than those of aggregating monomers. The NA-F fraction gave a broad distribution which was subfractionated into three fractions. The slowest, NA-F-α, was similar in profile to the NA-S-β fraction while the main peak, NA-F-β, distributed almost in the same fractions as the main peak, Agg-D1-β, of the aggregating monomers.

Immunological Differences of Subfractions—An antiserum raised against aA1-D1 from the rat chondrosarcoma and described in detail elsewhere (26) was used to compare the antigenicity of the different proteoglycan subfractions (Table I and Fig. 5). The purified aggregate and the Agg-D1 monomer fractions gave nearly the same ratio of antigenicity to hexuronic acid content, Table I, although the value for Agg-D1 was reproducibly slightly higher, perhaps because some antigenic sites accessible in monomer are masked in aggregate.

**Table 1**

| Antigenicity of Subfractions from aA1 | Hexuronic Acid | Antigenicity/Hexuronic Acid |
|--------------------------------------|---------------|-----------------------------|
| aA1-D1 equivalents (mg) | Hexuronic acid (mg) | Antigenicity/Hexuronic acid |
|--------------------------|-----------------|-----------------------------|
| Agg                      | 8.96            | 1.68                        | 5.33                        |
| Agg-D1                   | 8.49            | 1.51                        | 5.62                        |
| NA-S                    | 0.79            | 0.44                        | 1.80                        |
| NA-F                    | 0.82            | 0.25                        | 3.28                        |

*Antigenicity was determined by ELISA using anti-aA1-D1 antibodies with aA1-D1 as the standard.

**Fig. 5.** Antigenicities of aA1-D1 and aggregating monomers (a) and isolated hyaluronic acid-binding region and nonaggregated proteoglycans (b) for anti-aA1-D1 antibodies. Antigenicity was measured by the ELISA inhibition test. Antigenic samples in serial dilutions were incubated with anti-aA1-D1 antibodies (1:3000 dilution). The initial concentrations of antigens were 20 μg/mL. Aliquots with 2 μg of aA1-D1 were used to coat each well. HA, hyaluronic acid.

For a constant dilution of antiserum, equivalent dilutions on the basis of hexuronic acid contents of the original antigen aA1-D1 and of the Agg-D1 monomers gave almost identical absorption curves, Fig. 5a, indicating that almost all of the antigens recognized by the antiserum are present in the aggregating monomer population. The ratio of antigenicity to hexuronic acid content was much less for the NA-F proteoglycan fraction compared to Agg-D1 and even less for the NA-S fraction (Table I). The absorption curves for these fractions, as shown in Fig. 5b, are displaced greatly toward solutions with more concentrated solutes but show a similar range of absorption. When a purified sample of hyaluronic acid-binding region derived from clostripain digests of aA1 (25) was used to absorb the antiserum, the solid curve shown in Fig. 5b was observed. The range of absorption was only about 70% of that of aA1-D1, indicating that while most of the antigens recognized by the antiserum are directed against sites in the hyaluronic acid-binding region a significant proportion are not.

Further information was obtained when aliquots from the dissociative velocity gradients shown in Fig. 4 were tested for antigenicity (open circles, Fig. 4). There was greater antigenicity per hexuronic acid in slower sedimenting fractions of aggregate and Agg-D1 with the effect more pronounced in the fractions from aggregate. The larger nonaggregated proteoglycans in NA-S-β and the NA-F fractions had less antigenicity per hexuronic acid than did Agg-D1 proteoglycans, indicating some differences from aggregating monomers. The slowest sedimenting component, NA-S-α, did not exhibit any significant antigenicity in the absorption test. The results for peak fractions are summarized in Table II.

Because the antiserum contains a large proportion of antibodies directed against the hyaluronic acid-binding region, the results suggest that the greater ratio of antigenicity to
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Table II

Ratio of antigenicity to hexuronic acid in fractions from the glycerol dissociative zonal gradients

While CaSO₄ even at 1 M in concentration did not affect the ELISA, guanidine HCl gave an inhibitory effect. Thus, fractions from the dissociative gradients were precipitated with ethanol or dialyzed against 0.1 M Tris-HCl, 0.1 M sodium acetate, pH 7.2, to remove guanidine HCl before analyses.

| Subfraction no. | Hexuronic acid (μg/ml) | aA1-D1 (%aA1-D1/μg hexuronic acid) | Agg-α | Agg-β | Agg-D1-α | Agg-D1-β | NA-S-α | NA-S-β | NA-F-α | NA-F-β |
|-----------------|------------------------|----------------------------------|--------|--------|----------|----------|--------|--------|--------|--------|
| Agg-α (13)      | 26.1                   | 228                              | 8.74   | 2.18   | 0.084    |          |        |        |        |        |
| Agg-β (17)      | 39.8                   | 182                              | 4.54   | 1.26   | 0.032    |          |        |        |        |        |
| Agg-D1-α (13)   | 21.8                   | 182                              | 8.34   | 0.98   | 0.045    |          |        |        |        |        |
| Agg-D1-β (17)   | 42.9                   | 205                              | 4.78   | 0.92   | 0.022    |          |        |        |        |        |
| NA-S-α (6)      | 28.5                   | 5.5>                             | 0.12>  | 0.57   | 0.001    |          |        |        |        |        |
| NA-S-β (12)     | 27.7                   | 31.9                              | 1.15   | 0.11   | 0.004    |          |        |        |        |        |
| NA-F-α (12)     | 19.7                   | 44.6                              | 2.26   | 0.42   | 0.021    |          |        |        |        |        |
| NA-F-β (17)     | 29.8                   | 65.0                              | 2.28   | 0.70   | 0.024    |          |        |        |        |        |

**Notes:**

1. Absorption assay with anti-aA1-D1 as described in Table I.
2. Absorption assay with antiserum against the complex of hyaluronic acid-binding region-link protein and hyaluronic acid (1/8000) using purified hyaluronic acid-binding region (HABR) to coat the plate for ELISA.
3. Values are given as equivalents to the test antigen.
4. Numbers in parentheses indicate fraction numbers from gradients shown in Fig. 4.

Hexuronic acid for the smaller proteoglycans from the aggregate profile (Agg-α) reflects a higher ratio of hyaluronic acid-binding region to chondroitin sulfate in these molecules. The effect would be less pronounced for the Agg-D1 profile since a proportion of the smallest, low buoyant density monomers is not recovered in the Agg-D1 fraction. More direct evidence for this was obtained by testing peak fractions in an ELISA for the purified hyaluronic acid-binding region. Again, there was greater antigenicity per hexuronic acid in the smaller aggregating monomer fractions, Agg-α and Agg-D1-α (Table II). Antigenicity per hexuronic acid was less for the NA-S fractions and much less for NA-S-β. NA-S-α had no detectable antigenicity for the hyaluronic acid-binding region.

**Polydispersity of Agg-D1—** Fractions from the aggregate and Agg-D1 were analyzed for average chondroitin sulfate chain sizes after alkaline borohydride treatment by chromatography on Bio-Gel A-1.5m (Fig. 6). The starting aA1 sample had a peak value of $M_0 = 22,000$ for the chondroitin sulfate chains. A small peak, 2.4% of the hexuronic acid, eluting in the excluded fraction was shown to be hyaluronic acid. The average chondroitin sulfate chain size increased with an increase in the sedimentation rates for both aggregate and Agg-D1 fractions, i.e. $\alpha < \beta < \gamma$, indicating that the polydispersity in hydrodynamic size for aggregating monomers correlates to a large extent with differences in average chondroitin sulfate chain sizes in the subpopulations. The peak chain size for Agg-α was slightly shorter than that for Agg-D1-α, consistent with the removal of some aggregating monomer of smaller size and lower buoyant density in the dissociative CsCl gradients used to isolate Agg-D1.

In the accompanying paper (35), the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicate that Agg-α would contain some link protein. Therefore, only subfractions of Agg-D1 were used for further analyses. Amino acid analyses for subfractions of Agg-D1 showed that the compositions are very similar except possibly for a small relative increase in serine and glycine for Agg-D1-γ (Table III). The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of chondroitinase-digested samples in the accompanying paper (35) show a predominant, single core band across a size fractionation of aggregating proteoglycans with a smaller amount of a smaller core band which is more pronounced in fractions with smaller proteoglycan molecules. If proteoglycan monomers are selectively degraded in the tumor and portions of the chondroitin sulfate attachment region are removed, this could account for the reduced relative serine and glycine contents in Agg-D1-α and -β relative to Agg-D1-γ and for the presence of the smaller core band (see discussion in Ref. 35).

Sialic acid and glucosamine have been identified as constituents of oligosaccharides in proteoglycan monomers from the chondrosarcoma (7). The sialic acid and glucosamine contents decreased with an increase in hydrodynamic size for Agg-D1 fractions (Table IV). However, the relative amount of sialic acid per number of chondroitin sulfate chains was similar for

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4 The abbreviation used is: ELISA, enzyme-linked immunosorbent assay.

5 The peak was identified as hyaluronic acid by its susceptibility to Streptomyces hyaluronidase. Since about 70% of the hexuronic acid in aA1 is in aggregate, the proportion of hexuronic acid in hyaluronic acid for purified aggregate is 2.4%/0.7 = ~3.5%.

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Fig. 6. Gel filtration of glycosaminoglycans from subfractions. Details of Bio-Gel A-1.5m column chromatography were described under "Methods." Dotted lines show $V_o$, void volume of the column and $V_t$, total volume of the column. Parentheses indicate the average molecular size calculated from $K_v$ values of peaks under arrows. Hyaluronic acid (HA1) in the excluded fractions of aA1 and NA-S-α were 2.4 and 6.1% of the hexuronic acid, respectively.
each fraction, suggesting that the number of oligosaccharides is constant per core protein. These results are consistent with the primary difference in the subpopulations, being the result of differences in the average chondroitin sulfate chain size on different proteoglycans.

**Compositions of Nonaggregated Proteoglycan Fractions**—The smallest nonaggregated proteoglycan, NA-S-α, had chondroitin sulfate chains of similar size, 22,000, to those of the major aggregating monomers (Fig. 6). However, the distribution was skewed more toward smaller sizes as shown by the retardation of the hexuronic acid profile and the high baseline for the carbazole reaction around fractions 50-60. The NA-S-α also showed a high ratio of sialic acid per chondroitin sulfate chain, Table IV, suggesting that a large proportion of oligosaccharides is present. Further, NA-S-α had a different amino acid composition from the aggregating proteoglycans, with much higher glycerine and somewhat lower proline, alanine, and serine (Table III). These results plus the absence of antigeinicity suggest that NA-S-α is a distinctly different proteoglycan species from the major proteoglycans.

Subfractions from NA-F had almost identical amino acid compositions which appeared to be nearly the same as for aggregating proteoglycans, with slightly higher relative glycine content (Table III). Sialic acid contents per hexuronic acid, Table IV, were nearly the same as those for aggregating monomers. There was also a similar increase in average chondroitin sulfate chain size with increase in hydrodynamic size although the NA-F fractions had somewhat longer chains (Fig. 6). The results suggest that the subpopulations of NA-F may differ from each other primarily in average chondroitin sulfate chain sizes on different molecules.

The NA-S-β fraction had similar molecular features to NA-F-α in chondroitin sulfate chain size, Fig. 6, in sialic acid content, Table IV, in amino acid compositions, Table III, in size distribution on the glycerol gradients, Fig. 4, and in identical antigenic properties, Table II. The NA-S-β fraction, then, probably contains the same subpopulation of molecules as the NA-F-α fraction.

**DISCUSSION**

The present study introduces a new method for the isolation of pure aggregate. Centrifugation of aAl from the rat chondrosarcoma for 6 h on the zonal gradients yielded effective separation of aggregate from nonaggregated proteoglycans. This allowed isolation from purified aggregate of an Agg-D1 fraction in which all the monomers contain a functional hyaluronic acid-binding region. The advantages of this procedure are: (a) greater purity is obtained than for the differential sedimentation used by Heinegärd and Hascall (21), (b) more stable gradients are achieved at lower electrolyte concentrations than in the original procedure described by Franek and Dunstone (23) or the preparative procedure described by Hoffman (22), (c) hexuronic acid contents can be analyzed directly by automated methods which are not possible when halide is present, and (d) high loading capacities are possible (up to 100 mg of purified aggregate can be obtained in one preparative run). Other solvent conditions can be substituted for the 0.1 M Tris-HCl-0.1 M sodium acetate, pH 7.2, solvent used in this study as is demonstrated in the accompanying paper concerning mechanisms for aggregate formation (35).

Starting with the aAl fraction which contains intact aggregates isolated from the tissue without a dissociation step (11), 70-75% of the hexuronic acid was recovered in the purified aggregate. Monomers from the purified aggregate still showed a polydispersity in hydrodynamic size as shown by subfractionation on dissociative zonal gradients, clearly indicating that polydispersity is an intrinsic property of the aggregating monomers. The present study indicates that the polydispersity in hydrodynamic size is primarily related to a variation in the average size of chondroitin sulfate chains per core protein. However, we have shown in the accompanying paper (36) that monomers in the aggregate have two discrete core sizes, 80% large and 20% somewhat smaller. The latter population may represent a selective degradation product in which a piece near the outer end of the chondroitin sulfate attachment region has been trimmed off. Therefore, it is likely that two cores of different size will contribute secondarily to polydispersity in size and may account for the small differences in relative serine and glycine content for the Agg-D1 fractions.

Recent evidence from studies of biosynthesis of proteoglycans with chondrocytes from the rat chondrosarcoma in culture supports these conclusions. The core protein prior to adding chondroitin sulfate chains has been identified by double antibody immunoprecipitation using antibodies against the hyaluronic acid-binding region and has been shown to

**TABLE III**

Amino acid compositions of subfractions from glycerol density gradients

| Residues/1000 residues | Agg-D1 | NA-S | NA-F |
|------------------------|--------|------|------|
| Aspartic acid          | 56     | 63   | 61   |
| Throneine              | 98     | 95   | 106  |
| Serine                 | 134    | 135  | 145  |
| Glutamic acid          | 120    | 122  | 115  |
| Proline                | 87     | 90   | 75   |
| Glicine                | 131    | 136  | 144  |
| Alanine                | 73     | 69   | 66   |
| Valine                 | 66     | 65   | 60   |
| Isoleucine             | 34     | 34   | 34   |
| Leucine                | 79     | 77   | 78   |
| Tyrosine               | 25     | 16   | 17   |
| Phenylalanine          | 30     | 31   | 24   |
| Lysine                 | 15     | 18   | 17   |
| Histidine              | 15     | 13   | 18   |
| Arginine               | 36     | 34   | 38   |

**TABLE IV**

Relative contents of sialic acid

| Subfractions | Sialic acid/hexuronic acid | GlcN/GalN | Parameter of sialic acid/chondroitin sulfate chain | Parameter of GlcN/chondroitin sulfate chain |
|--------------|---------------------------|-----------|-------------------------------------------------|------------------------------------------|
| NA-S-α       | 0.030                     | 0.181     | 660                                             | 3982                                    |
| NA-S-β       | 0.025                     | 0.073     | 550                                             | 1606                                    |
| NA-F-α       | 0.027                     | 0.021     | 590                                             | 471                                     |
| NA-F-β       | 0.023                     | 0.021     | 510                                             | 298                                     |
| NA-F-γ       | 0.023                     | 0.023     | 620                                             | 452                                     |
| Agg-D1-α     | 0.035                     | 0.035     | 620                                             | 530                                     |
| Agg-D1-β     | 0.025                     | 0.016     | 540                                             | 471                                     |
| Agg-D1-γ     | 0.023                     | 0.018     | 590                                             | 471                                     |
| Agg-D1       | 0.026                     | 0.019     | 600                                             | 471                                     |

* Chain size analyses for NA-S-α, Fig. 6, showed hyaluronic acid in excluded fractions (61%), most, if not all, of which was derived from the exogenously added hyaluronic acid used to test for the ability of proteoglycans in the nonaggregated fraction to bind to hyaluronic acid (Fig. 3a). Therefore, the glucosamine content in NA-S-α, as well as in the other fractions, was corrected by subtracting the glucosamine due to hyaluronic acid.

* Calculated by multiplying the ratio (sialic acid/hexuronic acid) by the average molecular weight of chondroitin sulfate chains (see Fig. 6).

* Calculated by multiplying the glucosamine/galactosamine ratio by the molecular weight of chondroitin sulfate chains as in Footnote b.

* From the data of Oegema et al. (36).
contain a functional hyaluronic acid-binding region (28). In this case, the core protein prior to adding chondroitin sulfate gives a single, uniform band of high apparent molecular weight (~370,000) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In a separate study using the same chondrocyte system (37), the size polydispersity of the newly synthesized, aggregating proteoglycans was shown to be primarily related to differences in average chondroitin sulfate chain size per core protein in agreement with the results described in this paper.

The smallest proteoglycan (NA-S-α), which is about 11% of the aAl fraction, clearly represents a different population from aggregating monomers. It has different hydrodynamic size, lacks cross-reacting antigenicity, has a different amino acid composition, and has a relatively higher sialic acid content. These proteoglycans may be related to the class of small proteoglycans observed by other investigators in middle fractions from density gradients used to purify proteoglycans (38, 39).

The second population of nonaggregated proteoglycans (NA-S-β and Na-F-α to Na-F-γ), which are about 17% of the aAl fraction, is more difficult to define. NA-S-β and NA-F-α have a size distribution which overlaps Agg-D1. While the proteoglycans in this population share some antigenicity with the hyaluronic acid-binding region, the proportion per hexuronic acid is much less than for aggregating monomers, and they do not appear to interact with hyaluronic acid. Their amino acid compositions are similar. This population has many similarities with the nonaggregated proteoglycans isolated from bovine nasal A1 preparations (21) and may represent a distinct class or classes of proteoglycan from the aggregating population.

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REFERENCES

1. Hascall, G. K. (1980) J. Ultrastruct. Res. 70, 369-375
2. Hardingham, T. E., and Muir, H. (1972) Biochim. Biophys. Acta 279, 401-406
3. Hascall, V. C., and Heinegård, D. (1974) J. Biol. Chem. 249, 4232-4241
4. Heinegård, D., and Hascall, V. C. (1974) J. Biol. Chem. 249, 4250-4256
5. Gregory, J. D. (1973) Biochem. J. 133, 383-386
6. Heinegård, D. (1977) J. Biol. Chem. 252, 1980-1989
7. Lohmander, L. S., De Luca, S., Nilsson, B., Hascall, V. C., Capuco, C. B., Kimura, J. H., and Heinegård, D. (1980) J. Biol. Chem. 255, 6084-6091
8. De Luca, S., Lohmander, L. S., Nilsson, B., Hascall, V. C., and Caplan, A. I. (1980) J. Biol. Chem. 255, 6077-6083
9. Hascall, V. C., and Sajdera, S. W. (1970) J. Biol. Chem. 245, 4920-4930
10. Heinegård, D. (1972) Biochim. Biophys. Acta 245, 181-192
11. Faltz, L. L., Reddi, A. H., Hascall, G. K., Martin, D., Pita, J. C., and Hascall, V. C. (1979) J. Biol. Chem. 254, 1375-1380
12. Rosenberg, L., Hellmann, W., and Kleinschmidt, A. K. (1975) J. Biol. Chem. 250, 1877-1883
13. Thyberg, J., Lohmander, L. S., and Heinegård, D. (1975) Biochem. J. 151, 157-166
14. Kimura, J. H., Osdoby, P., Caplan, A. I., and Hascall, V. C. (1978) J. Biol. Chem. 253, 4721-4729
15. Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S., and Strider, W. (1976) J. Biol. Chem. 251, 6439-6444
16. Pasternack, S. G., Veis, A., and Breen, M. (1974) J. Biol. Chem. 249, 2296-2311
17. Reihaniian, H., Jamieson, A. M., Tang, L. H., and Rosenberg, L. (1979) Biopolymers 18, 1727-1747
18. Hardingham, T. E., Ewins, R. J. F., and Muir, H. (1976) J. Biol. Chem. 251, 127-143
19. Wieslander, J., and Heinegård, D. (1981) Biochem. J. 199, 81-87
20. Hardingham, T. E., and Muir, H. (1972) Biochem. J. 139, 505-511
21. Heinegård, D., and Hascall, V. C. (1979) J. Biol. Chem. 254, 927-934
22. Hoffman, P. (1979) J. Biol. Chem. 254, 11854-11860
23. Frankel, M. D., and Dunstone, J. R. (1967) J. Biol. Chem. 242, 3460-3467
24. Waasteson, Å. (1971) J. Chromatogr. 59, 87-97
25. Caputo, C. B., MacCallum, D. K., Kimura, J. H., Schrode, J., and Hascall, V. C. (1980) Arch. Biochem. Biophys. 204, 220-233
26. Rennard, S. I., Kimata, K., Gosalau, E., Barrach, H.-J., Wilczek, J., Kimura, J. H., and Hascall, V. C. (1981) Arch. Biochem. Biophys. 207, 399-406
27. Kimata, K., Barrach, H.-J., Brown, K. S., and Pennypacker, J. P. (1981) J. Biol. Chem. 256, 6961-6968
28. Kimura, J. H., Thonar, E. J. M. A., Hascall, V. C., Poole, A. R., and Reiner, A. (1981) J. Biol. Chem. 256, 7890-7897
29. Kimata, K., Okayama, M., Oohira, A., and Suzuki, S. (1974) J. Biol. Chem. 249, 1648-1653
30. Kimata, K., Irie, Y., Ito, K., Karasawa, K., and Suzuki, S. (1978) Biochem. Biophys. Res. Commun. 85, 1431-1439
31. De Luca, S., Heinegård, D., Hascall, V. C., Kimura, J. H., and Caplan, A. I. (1977) J. Biol. Chem. 252, 6600-6608
32. Rodbard, D. (1971) in Competitive Protein Binding Assays (Daughaday, W. D., and Odell, W. A., eds) pp. 204-208, Lippincott and Co., Philadelphia
33. Heinegård, D. (1973) Chem. Scr. 4, 199-201
34. Jurdian, G. W., Dean, L., and Roseman, S. (1971) J. Biol. Chem. 246, 430-435
35. Kimata, K., Hascall, V. C., and Kimura, J. H. (1982) J. Biol. Chem. 257, 3827-3832
36. Oegema, T. R., Hascall, V. C., and Dziewiatkowski, D. D. (1975) J. Biol. Chem. 250, 6151-6159
37. Pellini, S. A., Kimura, J. H., and Hascall, V. C. (1981) J. Biol. Chem. 256, 7883-7889
38. Stanescu, V., Maroteaux, P., and Sobczak, E. (1980) Biochim. Biophys. Acta 629, 371-381
39. Swann, D. A., Powell, S., and Somat, S. (1979) J. Biol. Chem. 254, 945-954
Swarm rat chondrosarcoma proteoglycans. Purification of aggregates by zonal centrifugation of preformed cesium sulfate gradients.

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