Variable Nature of Cartilage Proteoglycans*

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Cartilage proteoglycan aggregates are separated from collagen and other non-proteoglycan protein by preparative rate zonal sedimentation under associative conditions. Dissociative rate zonal sedimentation produces sedimented proteoglycan of lower protein content with a corresponding increase in the amount of less sedimentable protein-rich proteoglycan. An extensive number of sequential rate zonal sedimentations discloses that the process of disaggregation involves the separation of proteoglycans varying continuously in composition with no apparent discontinuities in distribution to indicate the presence of distinctively different macromolecules. The variations encompass proteoglycans of low protein content containing less than 2% keratan sulfate and proteoglycans with keratan sulfate as the predominant polysaccharide (present in concentrations greater than 2-fold that of the chondroitin sulfate) and more than a 10-fold increase in protein content.

Two distinctively different views of the aggregated proteoglycan systems of connective tissue ground substance are emerging to explain the experimental observations. The first involves a representative macromolecule containing all the structural features of the proteoglycan which aggregates because of external linking agents (1-3). Variations in composition within the aggregate represent, in this view, a microheterogeneity due to biological randomness, which may merely quantitatively change the degree of aggregation. Some macromolecules become nonrepresentative, because they do not participate in aggregating interactions (3) or do not disaggregate to representative subunits (4). Nothing has been revealed about these fractions to characterize them as chemically distinct species. A second view (5) describes the process of disaggregation as the separation of proteoglycans of continuously variable composition. The separation is effected because of interaction with the media used for the gradients (6).

Proteoglycan extracts contain small amounts of hyaluronic acid (3, 7), which interact hydrodynamically with selective proteoglycan fractions. However, the reaggregation observed in the analytical centrifuge after the remixing of dissociated proteoglycans has not been duplicated with the use of those hydrodynamically interacting fractions suggested as key components in the aggregation process (2, 3, 7, 8). A positive correlation should be observed if specific interacting components are being purified. Proteoglycans which do not separate from hyaluronic acid in dissociative processes (4) or which do not interact with hyaluronic acid after separation (3) are not necessarily qualitatively different macromolecules. There can be continuously variable numbers of interacting sites rather than the presence or absence of a specific site in the population of macromolecules. Variations in size and composition of proteoglycans would be expected to result in variations in concentration-dependent interactions with other agents. Thus, in a given ionic medium, the determinant as to which chondroitin sulfate oligosaccharides will bind to an anion exchange medium is a function of size and not of changes in binding site.

We do not imply that external reagents are not contributing factors in aggregation. The observations with hyaluronic acid (3, 7) conclusively establish that interactions occur with external polyanionic macromolecules excluding the competitive involvement of the massive numbers of anions (both sulfate and carboxyl) within the proteoglycan. This indicates an ordered structure which prevents uncontrollable self-aggregation because of both anionic and cationic interacting sites within the same macromolecules. However, the strong emphasis on external agents tends to diminish proteoglycan to a structurally homogeneous biological model with biologically inactive fractions as contaminants. That is precisely what the concept of subunit, or, alternatively, of the most representative fraction of a single population of molecules accomplishes.

In this report we attempt to place lower density proteoglycans into perspective as well authenticated components of the aggregate. The preparative rate zonal studies on which this report is based have demonstrated that the relative distribution of the proteoglycan macromolecules are markedly influenced by associative and dissociative systems, but there are no indications that the macromolecules are not continuously variable in composition.

MATERIALS AND METHODS

Bovine nasal septa and embryos were obtained fresh and iced from the slaughterhouse. The nasal septa and embryonic rudiments were cleansed, sliced, and freeze-dried. The dried tissues were milled in a

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Wiley mill and stored at room temperature until used. Portions of fresh tissue extracted immediately showed no differences in yields nor in characteristics of the extracted products in comparison trials.

Detailed references have been given (5, 6) to the methods for free flow electrophoresis, CsCl gradient centrifugation, sample collection, assays, and the simultaneous determination of amino acids (including hydroxyproline), glucosamine, and galactosamine.

Rate Zonal Centrifugation—Preparative rate zonal runs were normally made with the use of polystyrene tubes in a Spincos 50-1 apparatus by a Spincos 60 T angle rotor. Gradients were prepared from equal volumes of high and low density solvents in a linear gradient mixer and delivered to the top of the developing gradient with slow, nonturbulent flow. The gradient occupied approximately 80% of the tube volume. The sample to be centrifuged was dissolved at 4 to 10 mg/ml in the low density solvent diluted 1:1 with water. Alternatively, if a crude extract or a sample already in solution was used, it was adjusted to the same solvent concentration by dialysis. The sample was carefully layered on the gradient to fill the tube and was centrifuged in Beckman L or L 5-75 centrifuge, as described in the figure legends.

Solutions of 3 and 4 M guanidinium chloride and 8 M urea were prepared with the use of 0.04 M, pH 5, acetate buffer. The low and high density solvents were prepared by adding the solute to achieve the following densities (grams per ml): (a) NaCl, 1.03 and 1.19; (5) NaCl(3 M guanidinium chloride), 1.11 and 1.18; (c) NaCl (8 M urea), 1.15 and 1.22; (d) sucrose (3% NaCl), 1.08 and 1.15; (e) sucrose (4 M guanidinium chloride), 1.11 and 1.15; and (f) sucrose (8 M urea), 3% NaCl, 1.15 and 1.21.

After centrifugation, fractions were obtained with a Buchler Automatic Density Flow for automated nonturbulent aspiration from the surface. All fractions were thoroughly dialyzed before assay and recovery.

RESULTS AND DISCUSSION

Comparative Rate Zonal Centrifugation of Proteoglycan Extracts—The first rate zonal gradient centrifugation experiments reported for proteoglycan demonstrated a large degree of polydispersity (9). For maximal separation of polydisperse samples a shallow gradient of low density was suggested (10). However, our purpose was to develop large scale preparative methods to grossly separate proteoglycan, whether aggregated or disaggregated, from non-proteoglycan matter. Consequently, the minimum density of the gradient was kept relatively high (above 1.1 g/ml) to support a larger amount of the crude extract while the maximum was kept well below the 1.3 g/ml range at which isopycnic banding of the protein-rich proteoglycan fractions influences the distribution (6). In the equilibrated, high density gradients, where isopycnic banding effects predominate, molecular size has no influence, and single chain chondroitin sulfate and high density proteoglycan band together. Under preparative rate zonal conditions single chain chondroitin sulfate and even the multichain chondroitin sulfates isolated after trypsin treatment sediment more slowly and are well separated from the bulk of sedimented proteoglycan. Collagen, which is present in significant amounts in the crude extract, is absent or present in trace amounts in the sedimented proteoglycan. Nor does lysozyme, which interacts strongly with the polyanionic molecules, co-sediment. Consequently, any proteins linked purely by salt type linkages would be expected to separate on the gradients.

The distribution of recovered materials from the different gradient zones is shown in Fig. 1. These bar graphs represent eight independent, preparative gradients using crude extracts of bovine nasal septum and embryonic rudiments. The sedimented proteoglycans in the associative gradients (open bars) clearly have higher protein contents than do the major, dense fractions from preformed, associative, CsCl gradients (5). There is a very clear and sharp difference in the protein content of the major fractions in the gradients represented by the open bars and those represented by the solid bars (dissociative conditions). That is, the presence of 3 M guanidine or 8 M urea in the gradient causes the protein content of the major proteoglycan fraction to approximately halve with only a small decrease in the relative distribution of the total extract. Consequently, protein-rich material must be separating from proteoglycan sedimenting in the presence of guanidine or urea. This protein-rich material has all the characteristics of a protein-rich proteoglycan and contains the proteoglycans found in the less dense regions of CsCl gradients (see below).

Sequential Rate Zonal Sedimentation of Proteoglycans—Crude extracts of bovine nasal septum and embryonic rudiments were sedimented on NaCl gradients, and the major proteoglycan fractions were resedimented on NaCl gradients containing 3 M guanidinium chloride.

The distribution of the recovered fractions from the various zones and the protein contents are shown in the bar graphs of Fig. 2. As we have noted before (5), sedimentation in a gradient of a density below 1.3 g/ml gives rise to a pellet having a higher protein content than the soluble fraction. On gradients with maximum densities above 1.3 g/ml, pellets have lower protein contents than the contiguous soluble fractions (5). Consequently, the pellets represented in Fig. 2 resemble PPL5 material (11) except that most, if not all, of the collagen and salt-bound protein have been removed.

Table I clearly shows that the sedimented aggregates from the bottoms and pellets of the initial gradient are dispersed or disaggregated on the second gradient into more sedimentable, chondroitin sulfate-rich fractions and less sedimentable fractions of diminished chondroitin sulfate content but with significantly enhanced keratan sulfate and protein contents. The fractions represented in Fig. 2 and Table I are soluble fractions dialyzed before recovery. Significant amounts of insoluble protein separated on dialysis only from the upper zones of the gradients on which the total crude extracts were centrifuged. Assay of the solutions recovered from the gradi-
pattern is evident from the sequential rate zonal centrifugations described by Fig. 2. Virtually all of the collagen and nonsedimentable protein remains in Fractions 2AU and 2BU. In the second stage dissociative system the sedimented fractions are similar, but the more slowly sedimenting embryonic rudiment proteoglycans are richer in protein. It should be noted that because 2AB and 2BP represent the major fractions of the crude proteoglycans, Fractions 2CU and 2FU are the major respective less sedimentable fractions and present in comparable amounts with respect to the total crude proteoglycans. Similarly, while 2CB represents more of the total proteoglycan than 2DB, the opposite is true of 2EB and 2FB. These results indicate that differences in properties and consequent distributions of proteoglycans can be related to quantitative differences in composition of the proteoglycan aggregate population. The differences can be large and exert a real influence independently of whether or not hyaluronic acid is required for aggregation.

A more detailed analysis of sequential sedimentations in combinations of associative and dissociative gradients is shown in the data of Fig. 3 and Table II. These are from a single bovine nasal septum extract appropriately subdivided to rule out chance variations in extraction conditions and yields. A clear difference can be seen between the dissociative effects of 4 M guanidinium chloride in a gradient (Fig. 3A) and associative gradients of NaCl or sucrose (Fig. 3, B and C). The former system, in contrast to the latter, decreases the keratan sulfate as well as the protein content of the major sedimented proteoglycan fraction. Compare Fractions 3BB, 3CB with 3AB in Table II. Fractions 3AB and 3BB, Fig. 3, representing different stages of aggregation, were layered on identical gradients and simultaneously sedimented in the same rotor to rule out variations in the conditions of the run. It is quite obvious that in the absence of dissociative conditions the stability of the aggregate is maintained (Fig. 3E). It also becomes apparent that, after partial disaggregation in which proteoglycans of different composition separate from each other, further separation occurs even under associative conditions. The macromolecules which separate by virtue of decreased sedimentation (Fig. 3D) are retained under the same conditions of sedimentation in the totally sedimented aggregate (Fig. 3E). The separation on repeated sedimentation of proteoglycan fractions are reflected by characteristic changes in composition. The variations appear to be continuous. Compare the analysis in Table II of Fractions 3DF, 3DL, and 3DB.

The use of velocity sedimentation to compare the relative molecular size of proteoglycans is complicated both by the tendency for aggregation and by the large differences in composition of isolated subfractions. Velocity sedimentation reflects relative molecular size with any degree of certainty only in cases of constant composition. A continuous molecular size distribution is seen in further, short term, rate zonal studies with fractions similar to 3DL and 3DB. However, these fractions sediment faster than products of very similar composition produced by trypsin treatment, which, in turn, sediment considerably faster than single chain chondroitin sulfate. Urea appears to be an efficient dissociating agent as guanidinium chloride. Compare Figs. 3F and 3A, also Figs. 1A and 1B (solid bars). We have observed that 8 M urea containing NaCl extracts proteoglycan from freeze-dried cartilage as efficiently as does 4 M guanidinium chloride. Slight dilution of the urea has a large effect on the extraction efficiency, and 6 M
urea is a poor extractant. These results show that the protein, which is at least as variable as the different polysaccharides in the dispersed or disaggregated proteoglycan fractions, has a significant function in the aggregation process.

**Free Flow Electrophoresis of Proteoglycan Fractions**—The distribution of selected proteoglycan fractions after free flow electrophoresis are shown in Fig. 4. The samples selected were those with the minimum chondroitin sulfate content and largest protein content. For a proper perspective these should be compared with the free flow electrophoresis distribution curves for proteoglycans of varying protein content isolated after isopyknic equilibration on CsCl gradients (compare Fig. 4 in Ref. 5). Fig. 4A represents the distribution of Fraction 2AU (Fig. 2 and Table I). This fraction contains all the water-soluble material of a bovine nasal septum extract which has not sedimented to the bottom. Most of the protein is nonmigrating compared to proteoglycan. For reference, under the conditions of the electrophoresis, soluble collagen or albumin, as representative proteins, would collect in tubes 12 to 18. In contrast, the sedimented, pelleted fraction (Fraction 2AP of Fig. 2 and Table I) containing a higher protein content than the bottom soluble fraction migrates essentially completely as a protein-rich proteoglycan (Fig. 4B). Resedimentation of this fraction under dissociating conditions separates it into two major fractions (Fig. 2D and Table I; Fractions 2DU and 2DB). These differ 3-fold in protein content with corresponding changes in keratan sulfate and chondroitin sulfate. The electrophoretic mobility of Fraction 2DU (Fig. 4C) still characterizes it as a proteoglycan with the major protein-containing material migrating to the same range as the aggregate from which it was dissociated (Fig. 4B). The proteoglycan collected in tubes 23 to 46 of the electrophoresis represented by Fig. 4C was alkali-treated and then subjected to further electrophoresis (Fig. 4D). It is apparent that alkali labile chondroitin sulfate chains have separated. However, small amounts of chondroitin sulfate still remain with the protein, and it is still considerably more electronegative than pure reference proteins. This is characteristic of all proteoglycans investigated in this manner (5). Consequently, the bulk of the protein associated with protein-rich proteoglycan (Fig. 4 B, C, and D) is different than the bulk of the protein which separates readily by rate zonal centrifugation from proteoglycan (Fig. 4A). The electrophoretic distribution of the embryonic rudiment proteoglycan fractions parallels the nasal septum fractions with the notably higher protein contents having little effect on the relative mobility.

**CsCl Gradient Centrifugations**—The nasal septum proteoglycan fraction which separated under dissociative conditions as less sedimentable was equilibrated on a CsCl density gradient (Fig. 5A). A continuous variation of relative amounts of uronic acid and protein over the whole length of the gradient and a distribution of keratan sulfate throughout most of the range of the gradient is apparent. Free flow electrophoresis of the same fraction shows similar heterogeneity. Migration was to the collection tubes normal for proteoglycan, and the chondroitin sulfate and keratan sulfate overlapped completely,
but the peak tubes were not coincident. A variable population of proteoglycans in an aggregate could be dispersed by two independent factors in a dissociative rate zonal gradient. Those proteoglycans would separate to the greatest extent with either the largest change in density (in effect, the largest change in protein content) or the largest change in molecular weight with respect to the average density and molecular weight. Both effects appear to be operative in the separation of the fractions to account for the distributions. Analysis of the recovered low density and mid-density fractions are recorded in Table III. Thus, it is seen that nasal septum proteoglycan can be dissociated by sequential sedimentation to achieve low density and mid-density fractions are recorded in Table III. The least dense, protein-richest fraction was subjected to analytical free flow electrophoresis and has the characteristics of a protein-rich proteoglycan. Similar treatment of the least dense fraction from the gradient described in Fig. 5A (Fraction 5A1-4, Table III), which is even richer in keratan sulfate, shows the same characteristics on electrophoresis. Proteoglycan aggregate (from rate zonal sedimentation) shows the same continuous distribution on associating as on dissociative CsCl gradients. The essential difference is that more of the protein-rich components are included in the major dense fraction. The observed changes in distribution are entirely consistent with the increase in density of a polyanion which is present as cesium salt over the density of the same polyanion as a mixed cesium, guanidinium salt (6), permitting more of the population of macromolecules with continuously variable density to accumulate in the major dense fraction.

**Cs$_2$SO$_4$ Gradient Centrifugations—** A difficulty with equilibrium gradient distributions of proteoglycans in CsCl is that the cesium salts of most proteoglycans are sufficiently dense to accumulate in the densest portion of the gradient where CsCl rapidly approaches saturation. A Cs$_2$SO$_4$ gradient offers a radically different situation since proteoglycans equilibrate at much lower densities while proteins have only slightly reduced densities (6). Consequently, variations due to changes in composition from pure polysaccharide to pure protein can be visualized well within the range of the gradient with no accumulation at the extremes.  

![Graph](image)

**TABLE III**

| Fraction | Density range | Amino acid | Glucosamine | Galactosamine | Hydroxyproline residues/1000 amino acid residues |
|----------|---------------|------------|-------------|--------------|-------------------------------------|
| 5A1-4    | 1.50–1.59     | 43         | 310         | 170          | 4                                    |
| 5A5-9    | 1.62–1.77     | 21         | 480         | 210          | 0                                    |
| 5B1-2    | 1.45–1.53     | 45         | 230         | 310          | 1                                    |
| 5B5-6    | 1.73–1.81     | 7.8        | 41          | 110          | 0                                    |
| 5C2-4    | 1.26–1.33     | 66         | 130         | 100          | 2                                    |
| 5C5      | 1.38–1.41     | 45         | 230         | 310          | 1                                    |
| 5C6      | 1.46–1.48     | 9          | 62          | 920          | 0                                    |

*The symbols identify the individual fraction numbers or the range of fraction numbers combined (in Fig. 5, A, B, and C) corresponding to the recovered fractions.*

Analysis of fractions recovered from CsCl gradients described in Fig. 5. The observed changes in distribution are entirely consistent with the increase in density of a polyanion which is present as cesium salt over the density of the same polyanion as a mixed cesium, guanidinium salt (6), permitting more of the population of macromolecules with continuously variable density to accumulate in the major dense fraction.  

**A sedimented proteoglycan aggregate (Fig. 1, Fraction 1BB) was equilibrated on both a high and low density dissociative CsCl gradient. This aggregate still contained the low density proteoglycan used in the experiment described by Fig. 5A but is separated from the fraction numbers combined in (Fig. 5, A, B, and C) corresponding to the recovered fractions.**

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**FIG. 5.** CsCl centrifugations. Similar low density proteoglycan fractions with consistent analysis and electrophoretic mobility including sample 3FU (Table II) were combined, dissolved in CsCl (density 1.69 g/ml), and centrifuged in the Spinco 60 TI angle rotor at 50,000 rpm, 26°C, for 72 hours. Fractions were screened for protein (Δ–Δ), uronic acid (O–O), and hexose (Cl–Cl), and combined for isolation as indicated in Table III. The distribution is shown in A. The fraction corresponding to Fig. 1B, B(C), was dissolved separately in CsCl, 3 M guanidinium chloride (density 1.68 g/ml), and centrifuged in the same Spinco 60 TI angle rotor at 48,000 rpm, 14°C, for 65 hours. Fractions after dialysis against saturated NaCl and then water were assayed for protein (Δ–Δ) and uronic acid (O–O) and then combined for recovery as indicated in Table III. Distribution in the higher density gradient is shown in B and in the lower density gradient in C.  

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The observed changes in distribution are entirely consistent with the increase in density of a polyanion which is present as cesium salt over the density of the same polyanion as a mixed cesium, guanidinium salt (6), permitting more of the population of macromolecules with continuously variable density to accumulate in the major dense fraction.
rate zonal sedimentation (where velocity sedimentation predominates with minimal buoyancy equilibration influences) more closely reflects the analytical centrifugal results.

The process that has been outlined by the data presented is the separation of an aggregate of proteoglycan from extraneous collagen and other protein followed sequentially by a disaggregation which resolves the aggregate into subfractions with higher and lower contents of chondroitin sulfate, keratan sulfate, and protein; enrichment of a given component in one of the subfractions is balanced by its impoverishment in the other. In the crude cartilage extracts dealt with in this report, the polysaccharide contents are approximately 90\% chondroitin sulfate and 10\% keratan sulfate. We have been able to obtain chondroitin sulfate-rich fractions containing 1 to 2\% keratan sulfate and 3 to 4\% protein from both the nasal septum and embryonic rudiment extracts. These dense fractions cannot be resolved directly on dissociative CsCl gradients from the fraction designated A1-D1 (4) but have less than one-half the protein content and considerably less keratan sulfate. To complement these high density fractions, in the remaining portion of the proteoglycan (enriched in protein), the keratan sulfate content can be more than double the chondroitin sulfate content (see Table III). This again is a greater resolution than that achieved directly on dissociative CsCl gradients (4). At one time the problem of whether keratan sulfate and chondroitin sulfate were components of independent proteoglycans or were variable components of a family of proteoglycans was actively considered. Interpretations tended toward the latter case, but there is no reason why both solutions could not be correct if variations occur between 100\% of one component continuously to 100\% of the other. In any event, a very large degree of variation characterizes the components which achieve unity in a proteoglycan aggregate and which separate into different distributions of proteoglycans dependent on the limitations of the methods of separation.

REFERENCES
1. Hascall, V. C., and Sajdera, S. W. (1969) J. Biol. Chem. 244, 2384-2396
2. Heinegård, D., and Hascall, V. C. (1974) J. Biol. Chem. 249, 4250-4256
3. Hardingham, T. E., and Muir, H. (1974) Biochem. J. 139, 565-581
4. Heinegård, D. (1972) Biochim. Biophys. Acta 285, 181-192
5. Hoffman, P., Mashburn, T. A., Jr., Hsu, D., Diep, J., and Trivedi, D. (1975) Connect. Tissue Res. 3, 177-186
6. Mashburn, T. A., Jr., Hoffman, P., and Hsu, D. (1974) Biochim. Biophys. Acta 362, 366-374
7. Hascall, V. C., and Heinegård, D. (1974) J. Biol. Chem. 249, 4232-4241
8. Hascall, V. C., and Heinegård, D. (1974) J. Biol. Chem. 249, 4242-4249
9. Franek, M. D., and Dunstone, J. R. (1967) J. Biol. Chem. 242, 3460-3467
10. Churchill, L., Banker, G., and Cotman, C. W. (1973) Anal. Biochem. 56, 370-382
11. Pal, S., Doganges, P. T., and Schubert, M. (1966) J. Biol. Chem. 241, 4261-4266
12. Mashburn, T. A., Jr., and Hoffman, P. (1971) J. Biol. Chem. 246, 6497-6506
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J. Biol. Chem. 1975, 250:7251-7256.

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