Proteoglycans from Bovine Proximal Humeral Articular Cartilage*

(Received for publication, July 10, 1972)

LAWRENCE C. ROSENBERG, SUBHASH PAL, AND ROBERT J. BEALE

From the Departments of Orthopedic Surgery and Medicine, and the Rheumatic Diseases Study Group, New York University School of Medicine, New York, New York, 10016

SUMMARY

To provide methods applicable to the study of human articular cartilage, we have studied the extraction, fractionation, and physical characterization of proteoglycans from bovine articular cartilage. Two dissociative solvents, MgCl₂ and GuHCl, differ sharply in the amount of the proteoglycan extracted from articular cartilage and the macromolecular species present in the initially extracted crude proteoglycan. PG(MgCl₂), the crude proteoglycan extracted with 3 M MgCl₂, is obtained in a yield of 0.16 g per g of dry cartilage, and accounts for 50% of the uronate and 23% of the hexose of the whole cartilage. PG(GuHCl), the crude proteoglycan extracted with 4 M GuHCl, is obtained in a yield of 0.26 g per g of dry cartilage, and accounts for 81% of the uronate and 61% of the hexose of the whole cartilage.

On analytical ultracentrifugation in associative solvents, PG(MgCl₂) shows three components: a 4 S protein, a 16 S proteoglycan species, and a 56 S proteoglycan aggregate. The proteoglycan aggregate in either PG(MgCl₂) or PG(GuHCl) is completely and reversibly dissociable into smaller units without breaking covalent bonds, appears to represent a basic structural unit and has been called the proteoglycan subunit (PGS). PG(GuHCl) contains a high molecular weight complex, not present in PG(MgCl₂). The complex differs from the 70 S proteoglycan aggregate in size, as indicated by its dissociation in 3 M GuHCl but not in 3 M MgCl₂.

Analytical ultracentrifugation of the dissociated complex in 3 M GuHCl shows that a 2.7 S protein is a major component of the complex. None of the 2.7 S protein is detectable in preparations of PG(MgCl₂) or of the 70 S aggregate.

Two states of macromolecular organization of proteoglycans appear to exist in articular cartilage. The first, and lower level of organization is that of the 70 S proteoglycan aggregate. The second and higher level of organization is that of the complex formed by the noncovalent association of proteoglycans and collagen in the presence of the 2.7 S protein.

Proteoglycans comprising the ground substance of cartilage consist of chondroitin sulfate and keratan sulfate chains covalently bound mainly to serine and threonine residues of a protein core. Two or more species of proteoglycans, which differ greatly in molecular weights, can be isolated from cartilage ground substance (1-11). The proteoglycan species of lowest average molecular weight, not dissociable into smaller units without breaking covalent bonds, appears to represent a basic structural unit and has been called the proteoglycan subunit (PGS) (4, 5). PGS is polydisperse with regard to molecular weight and composition (12), the molecular weight increasing in proportion to the numbers and lengths (13) of the mucopolysaccharide chains attached to the protein core.

The higher molecular weight proteoglycan species are aggregates formed by the noncovalent association of PGS molecules with a glycoprotein called the glycoprotein link (GPL) (5). Two classes of aggregate forms have been shown: first order aggregates consisting of roughly 10 to 16 molecules of PGS radiating outward in starlike fashion from a central locus of association presumably representing GPL; and second order aggregates consisting of larger numbers of PGS molecules clustered around several loci of association (10, 11).

The hypothesis for the macromolecular organization of proteoglycans composed of PGS and the GPL is that the proteoglycan subunit is a basic unit and that it complexes with the glycoprotein link...
glycans described above has been constructed almost entirely from studies of proteoglycans from bovine nasal cartilage. The question now arises whether this concept can be further supported by the study of proteoglycans from other cartilages and established as generally valid, or whether important differences exist in the macromolecular organization of proteoglycans in different cartilages. To pursue this question and at the same time provide information and methods directly applicable to the study of normal and diseased human articular cartilage, we are studying proteoglycans from bovine articular cartilages. Fetal, immature, mature, and aging cartilage is readily available from the articular surfaces of several bovine diarthrodial joints, including the proximal humerus, distal humerus, proximal ulna, and occipital condyles. The work reported here deals only with proteoglycans from normal mature proximal humeral bovine articular cartilage.

**EXPERIMENTAL PROCEDURE**

**Materials**—Guanidine hydrochloride (GuHCl) "absolute grade" and CsCl “O.D. grade,” were obtained from Research Plus Laboratories, Inc. 2-(N-Morpholino)ethanesulfonic acid monohydrate (MES) was from Calbiochem. Other chemicals were of reagent grade.

**Analyses**—The methods used were: uronate (14), hexose (15), hexosamine (16), sialate (17), protein (18), hydroxyproline (19), and sulfate (20).

**Extraction Methods**—Humeri were removed from 1- to 2-year-old cattle immediately after slaughter. Cartilage slices were rapidly collected from the articular surfaces of the proximal humeri with scalpels and finely diced into 1-mm cubes. For large scale preparations 100 g of diced cartilage were stirred in 1 liter of 3 M MgCl₂-0.02 M MES, pH 5.8, or in 1 liter of 4 M GuHCl-0.02 M MES, pH 5.8, for 24 hours at 5°C. The extract was filtered through coarse sintered glass, then filter paper, and 3 volumes of cold ethanol were added. The precipitate was collected by centrifugation, washed three times with ethanol, then ether, and dried in a vacuum. For sequential extractions, the cartilage was resuspended in fresh solvent and stirred for further 24-hour periods.

Concentrations of MgCl₂, CaCl₂, and GuHCl for maximal extraction of proteoglycan were determined in small scale experiments. Diced fresh wet cartilage (2 g) was weighed into screw cap culture tubes (25 x 100 mm), to each of which 40 ml of solvent were added. The tubes were oscillated end over end on a Buchler test tube shaker for 24 hours at 5°C. Proteoglycan was precipitated from the filtered extract, washed, and dried as described above. Extracted uronate, hexose, and protein were determined and expressed as percentage of total tissue uronate, hexose, and protein.

**Equilibrium Density Gradient Centrifugation**—Two methods of density gradient centrifugation were used for fractionating the initially extracted crude proteoglycan, with fundamentally different results. The first method, corresponding to the quick, one-step method for preparing PGS used by Hascall and Sajdera (5), involves centrifugation of the crude extract in CsCl-GuHCl without prior removal of extraneous protein. Most of the proteoglycan from bovine articular cartilage is recovered from the bottom third of the gradient, free of aggregate, as PGS. However, GPL is present in the top third of this gradient mixed with several other components which obscure reaggregation studies utilizing analytical ultracentrifugation. Therefore, the one-step procedure is useful for the preparation of PGS but not as a first step in the isolation of GPL.

The following procedure for the one-step CsCl-GuHCl gradient provided approximately 1 g of PGS: 1.5 g of PG(MgCl₂) or PG(GuHCl) were dissolved in 250 ml of 4 M GuHCl-0.05 M MES, pH 6.3 at 5°C. The solution was brought to room temperature and the pH maintained at 6.3 while 178 g of CsCl were added slowly. The final volume of the solution containing 3.4 M CsCl, 3.2 M GuHCl, and 0.04 M MES at 20°C was 310 ml, enough to fill eight tubes of a Beckman 50.1 fixed angle rotor. Centrifugation was carried out in polyallomer tubes with high force cap assemblies at 40,000 rpm (128,000 x g) for 40 hours at 20°C. Each gradient was divided into six equal fractions by gently suctioning into common pools from the top of the gradient.

The four fractions from the top of the gradient were exhaustively dialyzed against distilled water at 5°C for 48 hours, then freeze-dried. The two fractions from the bottom of the gradient were dialyzed against 0.15 M potassium acetate, pH 6.3, for 48 hours at 5°C, precipitated with 3 volumes of ethanol, washed with ethanol and ether, and dried in a vacuum.

The second method of density gradient centrifugation was a modification of the two-step procedure described by Hascall and Sajdera (5). To avoid precipitation of the initially extracted crude proteoglycan, reduce manipulations to a minimum, and prepare proteoglycan aggregate under conditions least likely to cause degradative effects, the first step, involving centrifugation under associative conditions, in 4 M CsCl, was carried out as follows. Fresh, wet articular cartilage slices (50 g) were collected as rapidly as possible and added immediately without diluting to cold 3 M MgCl₂-0.02 M MES, pH 6.3 (250 ml), or to 3 M GuHCl-0.09 M MES, pH 6.3 (600 ml). The cartilage was extracted by slow stirring at 5°C for 48 hours. The extract was filtered through sintered glass, then filter paper, then dialyzed against 9 volumes of 0.15 M potassium acetate, pH 6.3, for 24 hours at 5°C, to re-form aggregate. The volume of the 3 M MgCl₂ extract doubled on dialysis. The dialyzed extract was brought to room temperature and to 230 ml of this solution, 209 g of CsCl were added slowly with gentle stirring while the pH was maintained at 6.3. If required, the final volume of the solution was adjusted to 310 ml at 20°C with additional extract. The final solution contained 4 M CsCl, ~3 mg of proteoglycan per ml, and its density at 20°C was 1.54 g per ml. Centrifugation was carried out at 20°C for 40 hours in a 50.1 rotor and the gradient was divided into six fractions, as described above. The top one-sixth of this gradient yielded a 4 S protein, lysozyme, and a small amount of collagen, while the bottom one-sixth contained the 16 and 70 S proteoglycans. Fractionation of the 3 M GuHCl extract gave similar results, except that components of the PP-H-like complex separated at the surface of the gradient as a thick precipitate which was removed with forceps before the gradient was divided into six fractions.

**Analytical Ultracentrifugation**—Sedimentation velocity experiments were carried out in a Spinco model E analytical ultracentrifuge at 48,000 rpm and 20°C, using double sector cells with 30-mm centerpieces. Solutions for analytical ultracentrifugation, viscometry, and partial specific volume determinations were prepared by dissolving dry proteoglycan samples in phosphate buffer containing 0.10 M KCl, 0.02 M KH₂PO₄, and 0.03 M KHPO₄ (pH 6.5), unless otherwise stated. Addition of solvent to dry samples and all dilutions were made by weight. Schlieren patterns were recorded on Kodak metallographic plates and read with a Nikon micro comparator.

**Viscosity Methods**—These were carried out as previously described (10) in a constant temperature bath maintained at 20 ± 0.01°C with a mercury-in-glass thermoregulator (Arthur H. Thomas Co., model 9655G) and a Beckman differential thermometer.
Yields and analytical data on crude proteoglycans isolated from bovine proximal humeral articular cartilage by each of four successive 24-hour extractions with 3 M MgCl₂ or 3 M GuHCl.

Table I: Yields and analytical data on crude proteoglycans isolated from bovine proximal humeral articular cartilage by each of four successive 24-hour extractions with 3 M MgCl₂ or 3 M GuHCl. The compositions of the whole dry cartilage and the cartilage residue following extractions are also shown. Yields are given in g dry cartilage.

| Electrolyte | Whole dry cartilage | 24 hrs. | 48 hrs. | 72 hrs. | 96 hrs. | Cartilage residue |
|-------------|---------------------|---------|---------|---------|---------|------------------|
| 3 M MgCl₂   | Yield g/g           | 1.08    | 0.96    | 0.88    | 0.80    | 0.72             |
| Uronate    | %                   | 10.3    | 10.1    | 9.9     | 9.7     | 9.5              |
| Hexosamine | %                   | 6.0     | 5.8     | 5.5     | 5.2     | 5.0              |
| Protein    | %                   | 52.5    | 51.5    | 50.5    | 49.5    | 48.5             |
| Sialate    | %                   | 14.5    | 13.5    | 12.5    | 11.5    | 10.5             |
| Sulfate    | %                   | 4.5     | 4.0     | 3.5     | 3.0     | 2.5              |
| Hydroxyproline | %             | 8.1     | 7.5     | 7.0     | 6.5     | 6.0              |

Partial specific volumes were calculated from solute and solution densities determined with a 10-ml Sprengel pycnometer.

RESULTS AND DISCUSSION

Extraction Studies—Table I, Part A, gives analytical data on whole dry bovine articular cartilage and on the crude proteoglycan, PG(MgCl₂), obtained by extraction of fresh, wet cartilage with 3 M MgCl₂, pH 5.8, at 5°C for 24 hours. The analytical data on the whole cartilage reflect the much lower chondroitin sulfate and keratan sulfate contents, and the higher collagen content of this tissue, compared with bovine nasal cartilage and bovine nucleus pulposus (2). However, the extremely low yield of proteoglycan extracted in 24 hours (0.130 g per g of dry cartilage) with 3 M MgCl₂ is due only in part to the lower proteoglycan content of the articular cartilage. Calculations indicate that only 41% of the uronate and 19% of the hexose are removed in a single 24 hour extraction with 3 M MgCl₂.

To determine whether most of the proteoglycan could be removed with 3 M MgCl₂ by increasing the extraction time, three additional 24-hour extractions of the cartilage residue were carried out (Table I, Part A). While the composition of the proteoglycan removed in successive extractions does not change, the yield of proteoglycan drops sharply after the first extraction, so that the amount removed in 96 hours (0.163 g per g) represents essentially the maximum extractable with 3 M MgCl₂. This product accounts for only 50% of the uronate and 23% of the hexose in the whole tissue. Further extraction of the cartilage residue by high speed homogenization in water yields little or no additional proteoglycan. Twenty-nine per cent of the uronate and 59% of the hexose resist exhaustive extraction with 3 M MgCl₂, followed by high speed homogenization, and remained in the cartilage residue.

Because of the relatively low yields with 3 M MgCl₂, extraction experiments were carried out with 4 M GuHCl. Table I, Part B gives yields and analytical data on the crude proteoglycan, PG(GuHCl), obtained by four successive 24-hour extractions of cartilage with 4 M GuHCl, pH 5.8, at 5°C. The product extracted in 96 hours again represents the maximal extractable proteoglycan, but the yield (0.255 g per g) is much greater than that obtained with 3 M MgCl₂. Eighty-one per cent of the uronate and 61% of the hexose are removed as extractable proteoglycan with 4 M GuHCl, and roughly 90% of the extractable mucopolysaccharide is removed in the first 24 hours.

When extracting bovine nasal cartilage, Sajdera and Hascall (4) found that solutions of MgCl₂, CaCl₂, and GuHCl each exhibited an optimal electrolyte concentration. For MgCl₂ and CaCl₂, the concentration range over which the extraction efficiency was high was extremely narrow (4). While 3 M MgCl₂ gives optimal yields from bovine nasal cartilage, yields from articular cartilage were relatively low. To determine whether different cartilages required different electrolyte concentrations for optimal yields, extractions were made with varying MgCl₂, CaCl₂, and GuHCl concentrations. Fig. 1 shows the percentage of total tissue uronate and hexose extracted as a function of the electrolyte concentration. The extraction efficiency curves for bovine articular cartilage closely resemble those established for bovine nasal cartilage. KCl solutions show no significant differences in extraction efficiency over the concentration range studied and remove only 5% of the uronate and 3% of the hexose. The optimal concentration of each dissociative agent for bovine articular cartilage is identical with that established for nasal cartilage: 3 M MgCl₂, 2 M CaCl₂, 3 M GuHCl. The lower extraction efficiency of 3 M MgCl₂ cannot be attributed to a less than optimal concentration for aggregate dissociation. Indeed, experiments involving analytical ultracentrifugation presented below indicate that aggregate is rapidly and completely dissociated by either 3 M MgCl₂ or 3 to 4 M GuHCl. The sharp and constant differences in the yields of crude protein polysaccharide obtained with 3 M MgCl₂ and 4 M guanidinium chloride suggests that the capacity of GuHCl to mobilize an additional third of the proteoglycan results from an effect on the cartilage matrix distinct from dissociation of aggregate into PGS and GPL. The mobilization of this additional pool of proteoglycan is accompanied by the release of roughly 6% additional protein (Table I, Part B).
FIG. 2. Sedimentation velocity patterns showing the components present in PG(MgCl) and in PG(GuHCl). The direction of sedimentation is from left to right. A, PG(MgCl), 6 mg per ml in 0.10 M KCl-0.03 M K2HPO4-0.02 M KH2PO4, pH 6.95, at 56 min; B, PG(MgCl), 5 mg per ml in 3 M MgCl, 0.05 M MES, pH 5.8, at 168 min. The proteoglycan aggregate is dissociated. The major component is the proteoglycan species whose sedimentation coefficient ($s_0$,) is 16.5 S; C, PG(MgCl), 5 mg per ml in phosphate buffer containing 4 mol GuHCl, pH 7.0, at 68 min. The proteoglycan aggregate is dissociated; D, PG(GuHCl), 6 mg per ml in phosphate buffer, pH 6.95, at 46 min.

FIG. 3. Concentration dependence of the sedimentation coefficients of a 60 S proteoglycan aggregate (Curve A), the 16 S proteoglycan species (Curve B), and the 4 S protein (Curve C) present in PG(MgCl). Each set of points represents the experimental determined values for $s$. The lines represent the best least squares fit of the experimental points. a, $s$ against $c$; b, In $s$ against $c$; c, $1/s$ against $c$.

cartilage matrix. Observations presented below suggest that this protein interacts with proteoglycans and collagen to form a high molecular weight complex similar to the product previously called PP-H (21-23) which is dissociated by and soluble in 3 to 4 M GuHCl, but insoluble in 3 M MgCl.

Sedimentation Velocity Studies—In our work with articular cartilage proteoglycans, sedimentation velocity studies have proved indispensable for determining the number of species present in a preparation, identifying species during the development of procedures aimed at their isolation, and demonstrating association or dissociation under changing solvent conditions. The sedimentation coefficients given are the values extrapolated to zero concentration, at 20°, in the phosphate buffer ($s_0$,) unless otherwise stated.

PG(MgCl) showed the following components (Fig. 2A); I, a slow moving component in relatively low concentration with a sedimentation coefficient of $\sim$ 4 S. This component consists almost entirely of protein and is separated together with collagen from the proteoglycan species by equilibrium density gradient centrifugation in CsCl without GuHCl (Table III); 2, a major component with a hypersharp boundary and a sedimentation coefficient of $\sim$ 16 S. This component is the proteoglycan species of lowest molecular weight present in PG(MgCl); 3, a fast moving component in lower concentration with a sedimentation coefficient ranging from 60 to 70 S in different preparations. This component is the main proteoglycan aggregate of bovine proximal humeral articular cartilage; 4, higher order aggregate forms are also demonstrable in PG(MgCl), but their concentration is too low to allow the determination of sedimentation coefficients.

The concentration dependence of the sedimentation coefficients of a 60 S aggregate, the 16 S proteoglycan species and the 4 S protein are shown in Fig. 3a. Each set of points represents the experimentally determined values for $s$. The lines represent the best fit of the experimental points as indicated by the value of the index of determination following regression analysis. The equation for the curve for the 60 S aggregate (Fig. 3a, Curve A) is best described by an exponential function, $s = s_0 \exp^{-kt}$, with an index of determination of 0.984 (Table II, Preparation I). The question arises whether the equation $s = s_0 \exp^{-kt}$ with a value for $k$ of 0.312 fits the concentration dependence function over the entire concentration range from 1 to 6 mg per ml. Any change in the value of $k$ with concentration would be reflected in a plot of In $s$ against $c$ as a change in the value of the slope. In Fig. 3b, Curve A represents a plot of In $s$ against $c$ for the 60 S aggregate. There is no change in the value of $k$ over this concentration range.

The following observations indicate that the 70 S aggregate is reversibly dissociable. Sedimentation velocity studies of initial 3 M MgCl extracts show the 16 S proteoglycan species in high concentration, the 4 S component in extremely low concentration, and no species with sedimentation coefficients greater than $\sim$ 16 S; on dialysis of the extract against an associative solvent, the 60 S proteoglycan aggregate is formed (Fig. 2B). The 60 S aggregate present in reassociated PG(MgCl) is immediately and completely dissociated by 3 M MgCl (Fig. 2B) or by 3 M GuHCl (Fig. 2C). Fig. 4, Curve B, shows the concentration dependence of $s$ for the proteoglycan species in 3 M MgCl. Extradution to zero concentration gave a value for $s_{0,0}$ of 16.5 S.

Fractionation of PG(MgCl) by Equilibrium Density Gradient Centrifugation in CsCl—PG(MgCl) was separated into six fractions by equilibrium density gradient centrifugation under associative conditions in 4 M CsCl-0.15 M potassium acetate, pH 6.3, for 40 hours at 40,000 rpm and 20°. Yields and analytical data
Fig. 6, A and B. Two components are shown, a slow moving component in high concentration with “Schlieren patterns of Fraction 1s from PG(MgCl2) are shown in and of the 16 S species and 70 S aggregate into Fraction 6. A gradient is a sharp separation of the 4 S protein into Fraction 1, proportion of Fraction 6 (Fig. 6C) shows mainly the 4 S component and heterogeneous rapidly sedimenting components in low concentration. Table II.

| Species | Preparation | s0 (Svedberg) | Equation | k | Index of Determination | Standard error | Concentration (mg per ml) |
|---------|-------------|---------------|----------|---|------------------------|---------------|---------------------------|
| Proteoglycan Aggregate | (1) PG(MgCl2) | 59.9 | s = 0 exp(-kx) | .312 | .984 | 1.46 | 6 |
| | (2) PG(MgCl2) | 69.1 | s = 0 exp(-kx) | .364 | .930 | 3.22 | 5 |
| | (3) PG(GuHCl) | 56.5 | s = 0 exp(-kx) | .246 | .975 | .947 | 7 |
| | Fraction 6 | 71.3 | s = 0 exp(-kx) | .498 | .986 | 1.27 | 3 |
| | EDG (CsCl) | 69.3 | s = 0 exp(-kx) | .520 | .987 | 1.29 | 3 |
| | from PG(MgCl2) | 68.4 | s = 0 exp(-kx) | .400 | .988 | 1.30 | 5.0 |
| 16 S Proteoglycan Species | (7) PG(MgCl2) | 16.7 | s = 0 exp(-kx) | .162 | .981 | .372 | 6 |
| | (8) PG(MgCl2) | 15.3 | s = 0 -kc | 1.60 | .974 | 2.50 | 5 |
| | (9) PG(GuHCl) | 17.9 | s = 0 exp(-kx) | .188 | .989 | .264 | 7 |
| | Fraction 6 | 15.8 | s = 0 exp(-kx) | .153 | .987 | 1.42 | 4.6 |
| | EDG (CsCl) | 15.0 | s = 0 -kc | 1.84 | .956 | 9.83 | 5.0 |
| | from PG(MgCl2) | 14.3 | s = 0 -kc | 1.84 | .956 | 9.83 | 5.0 |
| | (11) Fraction 6 | 16.8 | s = 0 exp(-kx) | .179 | .979 | .553 | 3 |
| | EDG (CsCl) | 16.8 | s = 0 -kc | 1.79 | .979 | .553 | 3 |
| | from PG(MgCl2) | 16.8 | s = 0 -kc | 1.79 | .979 | .553 | 3 |
| | (12) Fraction 6 | 16.8 | s = 0 exp(-kx) | .220 | .982 | .361 | 5.6 |
| | EDG (CsCl) | 16.8 | s = 0 -kc | 1.84 | .948 | 9.48 | 4.2 |
| | from PG(GuHCl) | 14.7 | s = 0 -kc | 1.84 | .948 | 9.48 | 4.2 |
| | (13) Fraction 6 | 15.8 | s = 0 exp(-kx) | .230 | .972 | .408 | 4.2 |

The appearance of Fraction 6 is shown in Fig. 6D. Comparison of the schlieren pattern with Fig. 2A shows that the 4 S component originally present in PG(MgCl2) has been removed. Two components are now present, the 16 S species and 70 S aggregate. Comparison of the areas under the schlieren patterns in Fig. 6D with Fig. 2A indicates that the proportions of the 16 and 70 S species in Fraction 6 have been significantly increased over those in PG(MgCl2). With the increase in the relative concentrations of these species in Fraction 6, the intrinsic viscosity of this product increases to >600 ml per g, as compared to 200 ml per g for PG(MgCl2), and a change in the concentration dependence of sedimentation coefficients of the 70 S aggregate is noted, shown in Fig. 7 and Table II (Preparations 4 and 5). The concentration dependence function is still best described by s = 0 exp(-kx); however, the value of k increases to 0.5 and k remains constant only at concentrations less than 3 mg per ml, as shown both by the plot of s against c in Fig. 7A and by the plot of ln s against c in Fig. 7B.

The 70 S aggregate present in Fraction 6 is completely and reversibly dissociable in 3 M MgCl2 or 3 M GuHCl, in accord with the observations of Hascall and Sajdera on bovine nasal cartilage. However, while dissociation in 3 M MgCl2 yields the 16 S species, dissociation in 3 M GuHCl yields a species with c0.9 = 10.5 to 11 S, which we designate the proteoglycan subunit (PGS). Sedimentation velocity patterns and the concentration dependence of the sedimentation coefficients (c0.9) of PGS in 3 M GuHCl, are shown in Fig. 6, E and F, and Fig. 4, Curve C, respectively.

High Molecular Weight, PP-H-like Complex—Thus far we have described only those components present in the crude proteoglycan extracted with 3 M MgCl2, PG(MgCl2), and their distribution on the fractions are given in Table III. From these data, the percentages of uronate, hexose, protein, and hydroxyproline in each fraction have been calculated and are presented in Fig. 5.

Seventy-five per cent of the dry weight of PG(MgCl2) is recovered in Fraction 6, which contains 94% of the uronate and 87% of the hexose, as 16 S proteoglycan species and 70 S aggregate in roughly equal amounts, highly purified by the removal of collagen and extraneous protein. Only 10% of the dry weight, 57% of the uronate, and 6% of the hexose were recovered in Fractions 2, 3, 4, and 5 together, comprising the middle two-thirds of the volume of the gradient. In Fraction 1 are recovered 16% of the dry weight of PG(MgCl2), over 72% of the hydroxyproline, 48% of the protein, but less than 1% of the uronate, and 5.5% of the hexose. On dialysis against water prior to freeze-drying, roughly one-half of Fraction 1 precipitates. This precipitate was collected by centrifugation, freeze-dried, and designated Fraction 1 R.

Analytical data on this fraction and on the water-soluble product recovered from the supernatant (1 S) are given separately in Table III.

Sedimentation velocity studies show that the main effect of the gradient is a sharp separation of the 4 S protein into Fraction 1, and of the 16 S species and 70 S aggregate into Fraction 6. Schlieren patterns of Fraction 1S from PG(MgCl2) are shown in Fig. 6, A and B. Two components are shown, a slow moving component in high concentration with c0.9 = 4.1 S and a fast moving component in low concentration with a sedimentation coefficient of ~6 S. No 70 or 16 S species is detectable. Fraction 1R (Fig. 6C) shows mainly the 4 S component and heterogeneous rapidly sedimenting components in low concentration.
shown in Fig. 8. However, PG(GuHCl) also showed another

The 2.7 S protein is recovered in Fraction 1P following equilibrium density gradient centrifugation in 3 \( \times \) M \( \text{CsCl} \). None of the 2.7 S protein is present in PG(MgCl\(_2\)) or in PG(MgCl\(_2\)) on equilibrium density gradient centrifugation in 4 \( \times \) M \( \text{CsCl} \). We now show that the crude proteoglycan extracted with 3 to 4 \( \times \) M GuHCl, PG(GuHCl), contains a high molecular weight complex, which differs in its chemical composition and physical properties from the 70 S aggregate, and appears to represent a higher level of macromolecular organization. The complex is similar in its composition, sedimentation, and solubility properties to the fraction previously called FP-II (21–23).

Sedimentation velocity studies on PG(GuHCl) (Fig. 2D) showed the 4 S protein, the 16 S species, and a proteoglycan aggregate in lower concentration and slightly smaller in size (56 S) than the aggregate present in PG(MgCl\(_2\)). The concentration dependence of sedimentation coefficients of these species are shown in Fig. 8. However, PG(GuHCl) also showed another component of far greater molecular weight than the 70 S aggregate. It is heterogeneous and does not produce a well defined boundary on sedimentation. Its presence in PG(GuHCl) will not be detected in sedimentation velocity studies at speeds (48,000 rpm) appropriate for the study of the 70 S aggregate, unless it is specifically looked for during acceleration to operating speed. It is then recognizable as a thick layer of packed solute that appears at the base of the cell during acceleration. The sedimentation characteristics of this high molecular weight complex present in PG(GuHCl) are well demonstrated in terms of the change in the thickness of the layer of packed solute at 26,000 rpm, at solute concentrations of 5 mg per ml, shown in Fig. 9a. Some of the schlieren patterns from which measurements of packed solute volume were made are shown in Fig. 10. Each pattern in Fig. 10 was made at 6 min (Fig. 9a, arrows) after reaching 26,000 rpm.

As shown by the curve labeled PG(MgCl\(_2\)) in Fig. 9a, and in Fig. 10A, very little of the high molecular weight complex is present in PG(MgCl\(_2\)); the slow gradual increase in packed solute

**TABLE III**

Yields and analytical data on fractions separated from crude proteoglycans PG(MgCl\(_2\)) and PG(GuHCl), by equilibrium density gradient centrifugation in 4 \( \times \) M \( \text{CsCl} \), 0.15 \( \times \) M potassium acetate, pH 6.3

| Fraction | IP | IS | IR | 2 | 3 | 4 | 5 | 6 |
|----------|----|----|----|---|---|---|---|---|
| Yield g/q | .03 | .07 | .07 | .02 | .01 | .02 | .74 |
| Uronate % | 0.1 | 1.1 | 3.2 | 4.1 | 4.2 | 10.1 | 16.5 |
| Hexose | 2.9 | 3.2 | 3.1 | 3.1 | 3.9 | 11.8 |
| Hexosamine | 3.1 | 4.6 | 5.9 | 7.3 | 8.8 | 20.2 | 24.7 |
| Protein | 85 | 87 | 85 | 77 | 72 | 27 | 16 |
| Sialate | 0.1 | 0.4 | 0.2 | 0.4 | 2.4 | 2.1 |
| Sulfate | 1.8 | 7.5 | 4.1 | 4.9 | 5.6 | 15.1 | 15.6 |
| Asp | 0.02 | 0.02 | 0.03 | 0.04 | 0.04 | 0.02 |
| Density g/ml | 1.44 | 1.44 | 1.48 | 1.54 | 1.61 |

**Fig. 4.** Concentration dependence of the sedimentation coefficients \( (s_{0,2}) \) of the 16 S proteoglycan species, the 10.5 S proteoglycan subunit, and the 2.7 S protein involved in the formation of the 600 S complex. Concentrations of the 600 S complex and of Fraction 1P are 1.33 times the values given by the concentration scale. 4, in phosphate buffer, the proteoglycan species isolated by equilibrium density gradient centrifugation in 3.4 \( \times \) M \( \text{CsCl} \)-3.2 \( \times \) M GuHCl has a sedimentation coefficient \( (s_{0,2}) \) of 16.2 S. 5, in 3 \( \times \) M MgCl\(_2\), the proteoglycan aggregate of PG(MgCl\(_2\)) is dissociated into a species \( (\beta) \) whose sedimentation coefficient \( (s_{0,2}) \) of 16.5 S. 6, in 3 \( \times \) M GuHCl, the proteoglycan aggregate is dissociated into the proteoglycan subunit \( (\alpha) \) whose sedimentation coefficient \( (s_{0,2}) \) is 10.5 S. In 3 \( \times \) M GuHCl, the 600 S complex is dissociated into two components. The fast moving component \( (\beta) \) with a sedimentation coefficient \( (s_{0,2}) \) of 10.6 S is identified as the proteoglycan subunit. \( D \), the slow moving component \( (\gamma) \) formed by dissociation of the 600 S complex in 3 \( \times \) M GuHCl is a 2.7 S protein. The 2.7 S protein is recovered in Fraction 1P (9) following equilibrium density gradient centrifugation of PG(GuHCl) in 4 \( \times \) M CsCl. None of the 2.7 S protein is present in PG(MgCl\(_2\)) or in the fractions separated from PG(GuHCl) by density gradient centrifugation.

**Fig. 5.** Percentages of uronate (solid bars), hexose (lined bars), protein (stippled bars), and hydroxyproline (cross-hatched bars), recovered in each of the six fractions separated from PG(MgCl\(_2\)) by equilibrium density gradient centrifugation in 4 \( \times \) M \( \text{CsCl} \).
FIG. 6. Sedimentation velocity patterns of the fractions separated from PG(MgCl₂) by equilibrium density gradient centrifugation in 4 M CsCl. The experiments were carried out under standard conditions, except in E and F. The direction of sedimentation is from left to right. A and B, Fraction IS, 3.10 mg per ml at 16 and 112 min. The sedimentation coefficients (s₂₀, w) of the major components are 4.1 and 6 S; C, Fraction 1R, 5.32 mg per ml at 40 min; D, Fraction 6, 4.17 mg per ml at 64 min, showing the 16 S proteoglycan species and the 70 S aggregate; E and F, Fraction 6, 5.30 mg per ml in 3 M GuHCl, pH 7.0, at two times. The proteoglycan aggregate is dissociated. The major component, whose sedimentation coefficient (s₂₀, w) is 10.5 S, is the proteoglycan subunit.

FIG. 7. Concentration dependence of the sedimentation coefficients of the 70 S proteoglycan aggregate (Curve A) and the 16 S proteoglycan species (Curve B) in Fraction 6 isolated from PG(MgCl₂) by density gradient centrifugation in 4 M CsCl.

FIG. 8. Concentration dependence of the sedimentation coefficients of a 50 S aggregate (Curve A), the 16 S species (Curve B), and the 4 S protein (Curve C) present in PG(GuHCl).

FIG. 9. a, change in the thickness of the layer of packed solute with time at 26,000 rpm and 20°C. The arrows indicate the times at which the schlieren patterns shown in Fig. 10 were made. b, movement of the boundary of the 70 S aggregate from the solution meniscus with time.

The 600 S complex was isolated by preparative ultracentrifugation of a solution containing 6 mg per ml of PG(GuHCl) in 0.5 M potassium acetate, pH 6.3, for 30 min at 30,000 rpm. The yield of the complex was 0.4 g per g of PG(GuHCl). It contained 9.4% uronate, 9.0% hexose, 45% protein, and 0.21% hydroxyproline. The second feature by which the complex is distinguished from the 70 S aggregate (Table III, Column 6) is its lower proteoglycan content and its much higher protein and collagen content. The change in packed volume resulting from sedimentation of the isolated complex is shown at the top of Fig. 9a and in Fig. 10C.

The third group of properties that sharply distinguish the 600 S complex from the 70 S aggregate are the differences in solubility of the two macromolecular species in various solvents and the capacity of these solvents to dissociate the components of each of the two species. The 70 S aggregate is readily soluble in aqueous solvents containing monovalent or divalent cations over a wide range of electrolyte concentrations. The 70 S aggregate is immediately and completely dissociated in either 3 M MgCl₂ or 3 M...
Fractionation of PG(GuHCl) by Equilibrium Density Gradient Centrifugation in CsCl—Contrastingly, the 600 S complex becomes progressively less soluble in aqueous solvents containing increasing concentrations of electrolytes other than GuHCl. The complex is essentially insoluble in and not dissociated by 3 M MgCl2. However, it is readily soluble in and dissociated by 3 M GuHCl. The changes in packed volume resulting from sedimentation of the complex in 3 M GuHCl are shown by the curve at the bottom of Fig. 9a and in Fig. 10f. The complex has been dissociated, and the early large increase in packed volume is absent.

To identify the macromolecular species involved in the formation of the complex in terms of their sedimentation coefficients, the isolated complex was studied in sedimentation velocity experiments in 3 M GuHCl at 56,000 rpm. The two species demonstrated are shown in Fig. 10, E and F. The concentration dependence of their sedimentation coefficients (s20, w) is shown in Fig. 4, Curves C and D. Based on its sedimentation coefficient (s20, w) of 10.6 S, we identify the fast moving component as PGs, and the slow moving component, whose sedimentation coefficient (s20, w) is 2.7 S, as a heretofore undescribed protein, which is involved in the noncovalent binding of the various proteoglycan species to collagen. The amount of collagen present in the complex (~2%) is too small to produce a clearly defined schlieren pattern. A fourth feature by which the 600 S complex is distinguished from the 70 S aggregate, is that one of its components, accounting for roughly one-half of its weight, is the 2.7 S protein.

Fractionation of PG(GuHCl) by Equilibrium Density Gradient Centrifugation in CsCl—Compared with the fractionation of PG(MgCl2), fractionation of PG(GuHCl) under identical conditions gives different results. First, a thick precipitate separates at the surface of the gradient, not encountered in the fractionation of PG(MgCl2). This precipitate was removed with forceps, dispersed in and dialyzed against 0.15 M potassium acetate, pH 6.3, precipitated with ethanol, washed, dried, and designated Fraction 1S. The gradient was then divided into six equal fractions. Yields and analytical data on the fractions are given in Table III. From these data, the percentages of uronate, hexose, protein, and hydroxyproline in each fraction were calculated and are presented in Fig. 11.

The most important effect of the gradient is a sharp separation of the 2.7 S protein, together with most of the collagen involved in the formation of the 600 S complex, into Fraction 1P, and of the 16 S and 70 S proteoglycan species, essentially free of collagen and extraneous protein, into Fraction 6. Fraction 1P, in which the proportion of 2.7 S protein and collagen is higher, and the proportion of proteoglycan is lower than in the 600 S complex, is relatively insoluble in associative solvents. To identify the macromolecular species present in Fraction 1P, sedimentation velocity experiments were carried out in 3 M GuHCl at 56,000 rpm. One major component was demonstrated, shown in Fig. 12, A, B, and C. Based on the correspondence of the concentration dependence of its sedimentation coefficients (Fig. 4D, ○) to that of the 2.7 S protein present in the 600 S complex (Fig. 4D, ○), we identify this major component in Fraction 1P as the 2.7 S protein. The separation of the 2.7 S protein into Fraction 1P by density gradient centrifugation of PG(GuHCl) under associative conditions in 4 M CsCl represents a useful first step in the isolation of this species.

While Fraction 1S from PG(MgCl2) shows two distinct components (4 and 6 S proteins) on analytical ultracentrifugation, Fractions 1S and 1R from PG(GuHCl) are more heterogeneous and show broad boundaries (Fig. 12, D and E). These findings suggest that 3 M MgCl2 selectively extracts a more limited number of cartilage components in purer form. We recommend Fraction 1S separated from PG(MgCl2) for the isolation of the 4 S protein.

The schlieren pattern of Fraction 6 from PG(GuHCl) is shown in Fig. 12F. Two components are now present, a 16.8 S species and a 68 S aggregate, essentially free of collagen, 4 S protein, 2.7 S protein, and 600 S complex, as indicated by the analytical data, sedimentation velocity, and packed volume studies. The concentration dependence of the sedimentation coefficients of these species is shown in Fig. 13. The proportion of aggregate is invariably greater in Fraction 6 from PG(MgCl2) than in Fraction 6 from PG(GuHCl). We therefore recommend Fraction 6 from PG(MgCl2) for the isolation of GPL.
FIG. 12. Sedimentation velocity patterns of the fractions separated from PG(GuHCl) by equilibrium density gradient centrifugation in 4 M CsCl. The sedimentation velocity experiments were carried out at 48,000 rpm and 20° in 3 M GuHCl in A, B, and C, and in the phosphate buffer in D, E, and F. The direction of sedimentation is from left to right. A, B, and C, Fraction 1P, 5.47 mg per ml at 16, 64, and 192 min. The major component is the 2.7 S protein; D, Fraction 1S, 7.02 mg per ml at 80 min; E, Fraction 1R, 7.36 mg per ml at 48 min; F, Fraction 1R, 3.84 mg per ml at 40 min.

TABLE IV

|       | 1   | 2   | 3   | 4   | 5   | 6   |
|-------|-----|-----|-----|-----|-----|-----|
| Yield (g/g) | 0.231 | 0.152 | 0.075 | 0.027 | 0.134 | 0.380 |
| Uronate (%)  | 1.8  | 3.9  | 10.2 | 13.5 | 18.9 | 19.5 |
| Hexose       | 4.8  | 6.0  | 9.2  | 12.2 | 11.4 | 12.0 |
| Hexosamine   | 6.4  | 9.1  | 19.1 | 24.5 | 27.0 | 30.3 |
| Protein      | 64.1 | 55.7 | 2.90 | 14.9 | 13.4 | 13.9 |
| Sialate      | 1.8  | 2.7  | 1.9  | 1.4  | 1.4  | 1.4  |
| Hydroxyproline | 0.61 | 0.41 | 0.11 | 0.036 | 0.022 | 0.017 |

Fractionation of PG(GuHCl) in CsCl-GuHCl—PG(GuHCl) was fractionated by density gradient centrifugation in 3.4 M CsCl, 3.2 M GuHCl, 0.04 M MES, pH 6.3, corresponding to the one-step method of Hascall and Sajdera (5). Yields and analytical data on the fractions are given in Table IV. On analytical ultracentrifugation under associative conditions in the phosphate buffer, Fraction 5 shows one component (Fig. 14) with a sedimentation coefficient (c_{20,w}) of 16.2 S (Fig. 4, Curve A). The molecular weight of this species calculated from a sedimentation coefficient of 15.8 S (Table II, Preparation 13) in the phosphate buffer, partial specific volume of 0.594 ml per g, intrinsic viscosity of 184 ml per g (Fig. 15), and a value of 2.12 for β in the Scheraga-Mandelkern equation (24), is 1.3 X 10^6. No aggregate is detectable over a concentration range from 0.9 to 4.2 mg per ml. In accord with the observations of Hascall and Sajdera (5), ref-
nation of aggregate does not occur, once GPL has been separated into the top of the gradient.

The work reported here described similarities and differences in the macromolecular organization of proteoglycans from bovine articular and bovine nasal cartilage. In addition, conceptual and operational guidelines have been established for studying the more complicated organization of proteoglycans from articular cartilage. Two different states of macromolecular organization of proteoglycans exist in articular cartilage. One level of organization is that of the proteoglycan aggregate, identical in its properties with the analogous species from bovine nasal cartilage in the following ways: (a) It is extracted from the articular cartilage by MgCl₂, CaCl₂, or GuHCl, each at an optimal electrolyte concentration identical with that for nasal cartilage; (b) it contains roughly the components of the complex are extracted from articular cartilage: S mot. By GuHCl; (c) the aggregate in the following ways: (a) It is extracted from the articular cartilage by MgCl₂, CaCl₂, or GuHCl, each at an optimal electrolyte concentration identical with that for nasal cartilage; (b) it contains roughly proteoglycan aggregate is dissociated by concentrated electrolyte solutions containing monovalent or divalent cations, and is completely and reversibly dissociable in concentrated solutions of electrolytes containing divalent cations or GuHCl; (d) equilibrium density gradient centrifugation in CsCl-GuHCl results in its dissociation and separation into GPL and the proteoglycan subunit, neither of which alone can reassOCIATE to re-form aggregate.

The second and higher level of macromolecular organization is that of the 600 S complex. The complex differs from the 70 S aggregate in the following ways: (a) it is of much larger size as indicated by its sedimentation properties; (b) it contains roughly 50% proteoglycan, 2% collagen, and 40% 2.7 S protein; (c) the components of the complex are extracted from articular cartilage by GuHCl but not by MgCl₂; (d) the isolated complex is incompletely soluble or insoluble in concentrated solutions of monovalent or divalent cations other than GuHCl. The complex is readily soluble in, and dissociated by 3 M GuHCl into the 2.7 S protein, the 10.5 S proteoglycan subunit, and collagen. Since the proteoglycan aggregate is dissociated by concentrated solutions of divalent cations, while the 600 S complex is stabilized by the same high salt concentrations, different nonequivalent bonds must be involved in the association of the 2.7 S protein, proteoglycan, and collagen from those involved in the binding of proteoglycan subunit to GPL. The properties of the nonequivalent bonds involved in the formation of the 600 S complex closely resemble those of hydrophobic bonds (25).

REFERENCES
1. Pal, S., Doganesc, P. T., and Schubert, M. (1966) J. Biol. Chem. 241, 4261
2. Rosenberg, L., Schubert, M., and Sanderson, J. (1967) J. Biol. Chem. 242, 4601
3. Franek, M. D., and Dunstone, J. R. (1967) J. Biol. Chem. 242, 3460
4. Szajera, S. W., and Hascall, V. C. (1969) J. Biol. Chem. 244, 77
5. Hascall, V. C., and Szajera, S. W. (1969) J. Biol. Chem. 244, 2384
6. Dunstone, J. R., and Franek, M. D. (1969) J. Biol. Chem. 244, 3054
7. Tsiganos, C. P., and Muir, H. (1969) Biochem. J. 113, 879
8. Tsiganos, C. P., and Muir, H. (1969) Biochem. J. 113, 885
9. Tsiganos, C. P., Hardingham, T. E., and Muir, H. (1971) Biochim. Biophys. Acta 229, 529
10. Rosenberg, L., Pal, S., Hessle, R., and Schubert, M. (1970) J. Biol. Chem. 245, 4112
11. Rosenberg, L., Hellmann, W., and Kleinschmidt, A. K. (1970) J. Biol. Chem. 245, 4123
12. Hascall, V. C., and Szajera, S. (1970) J. Biol. Chem. 245, 4920
13. Warteson, A. (1971) Biochem. J. 122, 477
14. Dische, Z. (1947) J. Biol. Chem. 167, 189
15. Yemm, E. W., and Willis, A. J. (1954) Biochem. J. 57, 508
16. Schloss, B. (1951) Anal. Chem. 23, 1521
17. Warren, L. (1956) J. Biol. Chem. 224, 1971
18. Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265
19. Woessner, J. F. (1961) Arch. Biochem. Biophys. 93, 440
20. Green, J. P., and Robinson, J. D. Jr. (1969) J. Biol. Chem. 235, 1621
21. Gerber, B. R., Franklin, E. C., and Schubert, M. (1960) J. Biol. Chem. 235, 2870
22. Rosenberg, L., Johnson, B., and Schubert, M. (1965) J. Clin. Invest. 44, 1647
23. Rosenberg, L., Johnson, B., and Schubert, M. (1969) J. Clin. Invest. 48, 543
24. Scheraga, H. A., and Mandelkern, L. (1953) J. Amer. Chem. Soc. 75, 199
25. Tanford, C. (1970) Advan. Protein Chem. 24, 1
