Proteinpolysaccharide Complex from Bovine Nasal Cartilage

A COMPARISON OF LOW AND HIGH SHEAR EXTRACTION PROCEDURES*

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SUMMARY

Two procedures for isolating 80 to 85% of the total hexuronate from bovine nasal cartilage as proteinpolysaccharide complex are described and compared. The first method, termed disruptive, extracts the complex by exhaustive high speed homogenization of the tissue in salt solutions of low ionic strength; proteinpolysaccharides used in most previous investigations were extracted similarly. The preparation is then purified by precipitation as the cetylpyridinium salt. The second method, termed dissociative, avoids shear by extracting proteinpolysaccharides into solvents containing optimal concentrations of various electrolytes and then utilizes equilibrium density gradient sedimentation to remove glycoprotein and soluble collagen. The effects of pH, temperature, and nature and concentration of electrolyte on the efficiency of dissociative extraction are presented; of the parameters investigated, only the type and concentration of electrolyte appear to be critical. Although both procedures yield products which exhibit bimodal distributions of sedimentation coefficients in the ultracentrifuge, the disruptive method denatures the macromolecules and decreases their average sedimentation coefficients. The faster sedimenting mode reversibly disaggregates under dissociative extraction conditions, suggesting that disaggregation is fundamental to the extraction process. Aggregation appears to be affected through strong ionic interactions; however, reduction and alkylation of cystine residues prevents reaggregation, suggesting that the interaction also depends on the conformation of protein moieties of the macromolecules.

Sulfated glycosaminoglycans, predominantly chondroitin 4-sulfate, constitute about 50% of the dry weight of 1- to 2-year-old bovine nasal cartilage. Early studies on the extraction of cartilage led Meyer and Smyth (1) to conclude that "the major portion of the cartilage is a protein salt of chondroitin sulfuric acid." Shatton and Schubert (2) reported the preparation from bovine nasal cartilage of a product consisting of chondroitin sulfate and noncollagenous protein and suggested that the protein and polysaccharide were combined. Malawista and Schubert (3) later described an extraction procedure which utilized high speed homogenization of the cartilage and allowed rapid isolation of the product, which they termed chondromucoprotein. Gerber, Franklin, and Schubert (4) devised a centrifugal fractionation scheme which allowed partition of chondromucoprotein into two fractions which they termed proteinpolysaccharides, abbreviated as PP-L (light) and PP-H (heavy), according to their sedimentation characteristics. Pal, Doganges, and Schubert (5) developed a more complex differential sedimentation method which allowed fractionation of PP-L into four preparations designated PP-L3, PP-L4, PP-L5, and PP-L6. The results to be presented here concern preparations which apparently encompass all the macromolecular species present in these various proteinpolysaccharide preparations; for this reason, we will describe the pool of sulfated glycosaminoglycans found in cartilage, together with the associated noncollagenous protein, as proteinpolysaccharide complex.

Soluble proteinpolysaccharide complex can be extracted from bovine nasal cartilage in yields representing up to 85% of the total tissue hexuronate, depending on the isolation and purification methods used. PPC contains 8 to 20% noncollagenous protein as well as varying amounts of keratan sulfate and, in general, traces of collagen. Shatton and Schubert (2) first suggested that the chondroitin sulfate and protein components of PPC are covalently linked. The nature of the linkage was determined by Gregory, Laurent, and Rodén (6), who showed it to be a glycosidic bond of xylose to a serine hydroxyl group. The xylose groups are at the reducing termini of unbranched chondroitin sulfate polymers with molecular weights near 20,000. The

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1 The abbreviations used are: PPC, proteinpolysaccharide complex; PPC(Gu) and PPC(Mg), PPC preparations obtained by extraction of cartilage with 4 M guanidinium chloride and 3 M MgCl2, respectively, and subsequent purification with a CsCl density gradient technique; PPC(CP), PPC obtained by homogenization of the cartilage in 0.15 M KC1 and subsequent purification with cetylpyridinium chloride; PP-L3, PPC purified by differential sedimentation techniques as described in Reference 5. Table I contains a summary of nomenclature.
which indicates that the ease and efficiency of PPC mobilization are described which allow facile preparations of undegraded macromolecules. Two dissociative isolation procedures and a comparison of PPC isolated by dissociative procedures with PPC support the hypothesis that protein conformations maintained by disulfide bonds are necessary for aggregation to occur. Com-

TABLE 1
Comparisons of disruptive and dissociative PPC isolations

<table>
<thead>
<tr>
<th></th>
<th>Disruptive</th>
<th>Dissociative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>Extract cartilage at room temperature on a magnetic stirrer with 15 volumes of (a) 4 M guanidinium chloride for 24 hours, or (b) 3 M MgCl₂ for 48 hours.</td>
<td>Homogenize cartilage three times in a total of 60 volumes of 0.15 M KCl-0.05 M Tris-HCl, pH 7, using a Servall Omni-Mixer operated at top speed on ice for a total of 1 hour.</td>
</tr>
<tr>
<td>Clarification</td>
<td>Filter with suction and the aid of 5% (w/v) Hyflo Super-Cel.</td>
<td>Add CsCl to a density of 1.69 g/ml and centrifuge to equilibrium. Isolate PPC from bottom third of gradient.</td>
</tr>
<tr>
<td>Purification</td>
<td>Precipitate twice as the cetylpyridinium salt from 0.3 M KCl. Precipitate twice as the calcium salt from 60% ethanol.</td>
<td>Precipitate as the cetylpyridinium salt from 0.3 M KCl.</td>
</tr>
<tr>
<td>PPC(CP)</td>
<td>(a) PPC(Gu)</td>
<td>PPC(L3)</td>
</tr>
<tr>
<td>PPC(L3)</td>
<td>(b) PPC(Mg)</td>
<td></td>
</tr>
</tbody>
</table>

The procedure used for isolation of PPC(L3) is not given here. See Reference 8.

most widely accepted model for PPC consists of a protein core with a number of chondroitin sulfate chains attached to the hydroxyl groups of serines within the polypeptide. The resulting macromolecules have molecular weights estimated at several hundred thousand to several million. Many workers have attempted to measure the molecular weight and hydrodynamic parameters of PPC, but these studies have not led to a satisfactory understanding of its physical properties. This is probably attributable in part to the heterogeneity and polydispersity inherent in PPC. However, studies for the most part have been performed on PPC extracted from the tissue by means of high speed homogenization, which can introduce shear artifacts. We will refer to procedures which utilize homogenization to prepare PPC as disruptive isolations.

This communication describes a new extraction technique which gives high yields of PPC after gentle agitation of tissue slices in high ionic strength solutions. Evidence is presented which indicates that the ease and efficiency of PPC mobilization from the cartilage depends on the ability of the extracting medium to reverse specific aggregation of PPC in situ; for this reason, procedures based on this technique will be referred to as dissociative isolations. Results obtained after reduction of PPC support the hypothesis that protein conformations maintained by disulfide bonds are necessary for aggregation to occur. Comparisons of PPC isolated by dissociative procedures with PPC isolated in comparable yields by a disruptive procedure indicate that disruptive methods denature and depolymerize the macromolecules. Two dissociative isolation procedures and a subsequent cesium chloride density gradient purification step are described which allow facile preparations of undegraded PPC free of contaminating glycoprotein and collagen. PPC prepared in this way contains 8% protein and exhibits bimodal distributions of sedimentation coefficients when analyzed in the ultracentrifuge. A reduction and alkylation scheme is also presented by which a centrifugally unimodal PPC which represents 85% of the tissue hexuronate can be prepared. This reduced and alkylated preparation, in which there is no possibility of shear-induced artifacts, and in which all capacity for aggregation has been eliminated, should prove to be a useful PPC on which to make physical measurements.

EXPERIMENTAL PROCEDURE

Materials—Guanidinium chloride was prepared from Eastman guanidinium carbonate according to the method of Kawahara and Tanford (7). S-Methylisothiouronium chloride was prepared from the sulfate salt with HCl-barium chloride and recrystallized once from methanol before use. Dithiothreitol was obtained from Calbiochem. A. D. Mackay, Inc. (New York, New York), supplied 99.95% cesium chloride. Cetylpyridinium chloride (hexadecylpyridinium chloride) came from Matheson Coleman and Bell (East Rutherford, New Jersey). All other chemicals were reagent grade and were used as received.

Nasal septa from 1- to 2-year-old cattle were obtained from an abattoir within 1 hour of slaughter, freed of adhering noncartilaginous tissue and perichondrium, and rinsed with 0.9% NaCl solution at 4°C. The septa were then sliced with the aid of a Stanley Surform. Slices prepared in this manner were about 0.5 mm thick and were stored until use at −15°C.

Analyses—Hexuronic acid was determined by the modification of Bitter and Muir (8) of the carboxylate method of Dische (9); insoluble hexuronate-containing samples were first dispersed by heating at 100°C in 3 N H₂SO₄ for 30 min. Control experiments with PPC showed no detectable loss of hexuronate as a result of this treatment. Total hexosamines were determined by the Blix modification (10) of the method of Elson and Morgan (11). Hexosamine ratios and amino acids were determined on the amino acid analyzer. Hydrolysis conditions were, for hexosamines, 4 N HCl at 100°C in sealed tubes for 8 hours, and for amino acids, 6 N HCl in sealed and evacuated tubes for 20 hours. Moisture contents were determined by drying under vacuum for 3 hours at 78°C over P₂O₅.

Disruptive PPC Isolations—PP-L3 was prepared from cartilage slices according to the method of Pal et al. (5).

The procedure used for isolation of the cetylpyridinium chloride-purified PPC preparation (PP(CP)) is outlined in Table I under the heading “Disruptive.” The extraction step was very similar to that of a recent modification (5) of the original disruptive procedure developed by Malawista and Schubert (5). Notable differences include the use of fresh or frozen cartilage slices rather than dried cartilage powder, and the use of the Servall Omni-Mixer rather than the smaller VirTis 45 homogenizer. The clarification and purification steps were points of departure from the typical disruptive scheme. The extracts were clarified by vacuum filtration with the aid of 5% (w/v) Hyflo Super-Cel and made 0.3 M in KCl by addition of solid KCl. PPC was then selectively precipitated from the extraneous cetylpyridinium salt by the addition of 2.5 moles of cetylpyridinium chloride (as a 0.5% (w/v) solution in 0.3 M KCl) per mole of hexuronate present in the extract. The cetylpyridinium salt was collected by centrifugation at 2000 × g for 5 min, washed with 0.3 M KCl,
and dissolved in 50 volumes of 2.5 M \( \text{CaCl}_2 \). PPC was precipitated as the calcium salt by the addition of 2 volumes of ethanol. Ethanol precipitation from 2.5 M \( \text{CaCl}_2 \) was repeated to ensure complete replacement of the cetylpyridinium cation with calcium. The PPC was then washed with ethanol and ether and dried under vacuum over anhydrous \( \text{CaSO}_4 \). All steps up to the addition of cetylpyridinium chloride were performed at 4°C; all subsequent steps were at 20°C because of the low solubility of the cationic detergent at the lower temperature.

**Dissociative Extraction Experiments**—The effects of pH, temperature, and type and concentration of various agents on the extraction of hexuronate from the cartilage slices were investigated in the following manner. Weighed samples of approximately 1 g of cartilage slices were placed in screw cap culture tubes (25 x 150 mm). Aliquots of 20 ml each of appropriate extraction solutions were then added. The tubes were capped and agitated by tumbling end over end 25 times per min. The hexuronate contents of the extracting media were determined at various times and the results were tabulated as the extraction efficiency, i.e., the percentage of the total tissue hexuronate extracted.

**Dissociative PPC Isolations**—PPC from two dissociative extraction procedures was purified and investigated in some detail. Optimal extraction conditions as determined from the results of experiments described in the previous section were used. The two procedures are outlined in Table I under the heading “Dissociative.”

Guanidinium chloride-extracted PPC (PPC(Gu)) was prepared by extracting cartilage slices with 15 volumes of 4.0 M guanidinium chloride, 0.05 M Tris-HCl, pH 7.5, at room temperature with magnetic stirring for 24 hours. Magnesium chloride-extracted PPC (PPC(Mg)) was prepared by extracting slices with 15 volumes of 3.0 M MgCl\(_2\), unbuffered (pH 5 to 6), at room temperature with magnetic stirring for 48 hours. Purification of either PPC(Gu) or PPC(Mg) from this point was performed in the same way. The extracting solution was freed of slices and other particulate matter by vacuum filtration with the aid of 5% (w/v) Hyflo Super-Cel. The filter cake was sucked dry and discarded without washing. The filtrate was dialyzed against 200 volumes of 3.0 M Tris-HCl, pH 7.5, at room temperature with magnetic stirring for 24 hours.

**Analytical Ultra-centrifugation**—Analytical sedimentation velocity experiments were performed at 20°C in a Spinco model E ultracentrifuge with the use of interference optics and a rotor speed of 55,500 rpm. The camera lens was focused two-thirds of the height of the solution column above the lower window. Dilute PPC solutions (0.23% or less) were investigated in cells equipped with 30-mm Epon double-sectored centerpieces and sapphire windows. A Gaertner comparator was used to measure the fringe displacement as a function of radial distance on Kodak type II-G plates exposed at various times after initiating an experiment. The data were then analyzed with a Control Data 160G computer in order to calculate \( g(s) \) distribution functions of sedimentation coefficients, defined as follows.

\[
g(s) = (1/c_0) \frac{dc}{ds} (1)
\]

where \( c_0 \) is the loading concentration, \( c \) is the concentration at a given radial distance and time, and \( s \) is the sedimentation coefficient associated with that radial distance and time. In terms of the parameters actually measured, the function has the following form.

\[
g(s) = \frac{\omega^2 r^2}{A_0^2} \frac{dr}{dr} \left( \frac{dn}{dr} \right) \left( \frac{1}{n_0} \right) (2)
\]

where \( \omega \) is the angular velocity of the rotor in radians per second, \( t \) is the time in seconds (corrected for acceleration), \( r \) is the radial distance in centimeters, \( n_0 \) is the radial position of the air-solution meniscus, the ratio \( (r^2/n_0^2) \) is a correction for radial dilution, \( dn/dr \) is the incremental fringe displacement with respect to radial distance, and \( n_0 \) is the total fringe displacement attributable to solute. The methods used for calculation were essentially those of Schumaker and Schachman (13), but, whereas they determined \( A_0 \) from plots of concentration with respect to radial distance, we used computer methodology to evaluate \( dn/dr \) by differentiation of second order least squares polynomials fitted to groups of 6 to 10 \((n, r)\) points. A theoretical treatment of this method is given by Williams et al. (14); the first application to disperse polysaccharide systems was by Williams and Saunders (15).

Although the \( g(s) \) technique provides considerable information concerning the nature of polydisperse polyelectrolytes such as PPC, the technique does reflect the nonideality of these solutes at finite concentrations and care must be exercised in interpreting the calculated profiles. A detailed discussion of the limitations of the techniques will be presented below.

**Effects of Reduction on Centrifugal Properties**—Experiments to determine the effect of reducing agents on the \( g(s) \) profiles were performed as follows. A centrifuge cell was loaded with 1.0-ml aliquots of an untreated PPC solution and solvent, and a \( g(s) \) analysis was computed from data obtained in the usual way. Without disassembly of the cell, 10 \( \mu l \) of 1.0 M dithiothreitol were added to the solution side; reduction was allowed to proceed in the cell before redetermination of the \( g(s) \) profile.

**Reduction and Alkylation of Dissociatively Extracted PPC**—Satisfactory conditions for reduction and alkylation of PPC were found. Extent of alkylation was determined by measuring S-carboxymethylcysteine on the amino acid analyzer after careful anaerobic hydrolysis (16). The dissociative cartilage extract was brought to the following solvent conditions, either by dialysis or the addition of solid Tris base 4 M guanidinium chloride 0.1 M Tris-HCl, pH 8.5. The solution was purged with nitrogen, and dithiothreitol was added to a concentration of 10 mM; reduction was allowed to proceed anaerobically at 37°C for 4
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various electrolytes for 20 hollrs. Conditiks: 20 ml of solution per g of cartilage slices at 25° with gentle agitation as explained in the text. Guanidinium chloride (X—X), LiCl (O—O), and CaCl₂ (O—O) were buffered with 0.05 M Tris-HCl at pH 7.5. MgCl₂ (O—O) solutions were unbuffered; their pH varied between 5 and 6.

Iodoacetamide was then added to a final concentration of 40 mM; alkylation proceeded with stirring in the dark for 16 hours at room temperature. The solution was dialyzed against 10 volumes of 0.1 M Tris-HCl, pH 7.5, and purified by CsCl equilibrium density gradient centrifugation as described above.

RESULTS

The results will be presented in three parts. First, parameters important for effective mobilization of PPC from the cartilage with dissociative methods will be described. Second, some physical and chemical characteristics of PPC isolated by two disruptive methods will be presented to provide a basis for comparison to those of dissociatively extracted PPC, which will be presented in the third part.

Dissociative Extraction Experiments—Fig. 1 shows the percentage of total tissue hexuronate extracted from cartilage slices as a function of concentration of a number of electrolytes after 20 hours of agitation at room temperature. All the electrolytes exhibited optimal concentrations for extraction efficiency, although the optimum differed in each case. Additionally, the sharpness of the curves varied, being broad for guanidinium chloride but rather narrow for the inorganic salts. The rate of extraction was greater for the optimal guanidinium chloride concentration than for optimal inorganic electrolyte concentrations, although the maximum tissue hexuronate extracted in each case reached the same value, approximately 85% of the total. This is shown in Fig. 2 for 4 M guanidinium chloride and 3 M MgCl₂ as contrasted with a 0.15 M KCl control. Note that both dissociative solvents remove more than 4 times as much hexuronate as the control, and do so with little possibility for bacterial contamination, autolysis, or rupture of covalent linkages. Also, 85% of the tissue hexuronate is the maximum which can be extracted by exhaustive, repetitive homogenization in the disruptive preparation of PPC(CP). Further homogenization of slices extensively extracted with 4 M guanidinium chloride in the same solvent failed to solubilize a significant amount of the residual hexuronate. One additional interesting observation is that the macroscopic physical appearance of the slices was unchanged by the extraction process even though the amount of PPC solubilized at optimal solvent conditions accounts for almost half the dry weight of the tissue.

Increasing the temperature from 2° to 40° increased the rate of PPC extraction in 4 M guanidinium chloride without affecting the maximum amount solubilized. At 40°, 85% of the hexuronate was extracted within 5 hours, whereas 48 hours were required for comparable yields at 2°. No apparent differences in the centrifugal patterns of PPC isolated at any of these temperatures were found. Extraction at optimal guanidinium chloride concentrations did not proceed well below pH 4.0, but between pH 6 and pH 9 neither the rate of extraction nor the final yield varied greatly.

Of the electrolytes tested, guanidinium chloride was most effective in mobilizing PPC from the tissue slices; its optimum concentration range was broader and the mobilization it induced...
was faster than that of any of the organic electrolytes. Two structural analogues of guanidinium were then investigated; the results are shown in Fig. 3. The charged analogue S-methyl isothiouronium proved as effective as guanidinium up to the limit of its solubility at 4.7 M. Urea, carrying no charge, was less effective even at the highest concentration tested, 8 M. However, urea proved to have a synergistic effect when used at 6 M in combination with increasing concentrations of CaCl₂. The narrow range of optimally efficient CaCl₂ concentrations was broadened by the addition of urea and displaced toward lower ionic strengths.

**PPC from Disruptive Isolations**—Two PPC preparations were studied, PP-L3 as described by Pal et al. (5) and cetylpyridinium-purified PPC(CP) prepared as outlined under “Experimental Procedure.” Analytical data for these preparations are included in Tables II and III. Note that although PP-L3 has a lower protein content than PPC(CP), it represents only 25% of the tissue hexuronate while the latter represents 85%. The hydroxyproline content of both is negligible (less than 0.05%), indicating virtual absence of collagen. Franek and Dunstone (17) have used equilibrium density gradient centrifugation to remove glycoprotein from unfractionated PP-L. Application of their techniques to PPC(CP) indicated the absence of glycoprotein at the 1% confidence level.

In the past, authors have observed that PPC preparations are polydisperse (18, 19); however, no quantitative estimation of the extent of solute dispersity inherent in these preparations has been presented. The g(s) distribution technique allows such estimations to be made from ultracentrifugal data and is a particularly appropriate physical method for comparing protein-polysaccharide complexes isolated using different methods. Apparent distributions for PP-L3 and PPC(CP) at two different loading concentrations are compared in Fig. 4, a and b. Both preparations were highly disperse and showed marked concentration effects even at high ionic strength and high dilution. In addition, the profile for PPC(CP) was bimodal; the slower sedimenting mode predominated, representing 70 to 80% of the sample weight. These results in combination with those derived from other loading concentrations, which for the sake of clarity are not included in the figure, allowed an extrapolation of the s values corresponding to the centers of the modes to zero con-
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Fig. 5. Effects of solute concentration on the mean sedimentation coefficients of the modes of three PPC preparations. Conditions were as in Fig. 4. The single mode of PP-L3 (X—a—X) extrapolates to a mean value of 14 S at infinite dilution. The corresponding values for the slower sedimenting modes of PPC(CP) (——O) and PPC(Mg) (——■) are 21 and 25 S; the values for the faster sedimenting modes of PPC(CP) (—●—) and PPC(Mg) (■—■) are 59 and 100 S.

Table III

Amino acid composition of PPC preparations

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>PP-L3</th>
<th>PPC(CP)</th>
<th>PPC(Gu)</th>
<th>PPC(Mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>73</td>
<td>81</td>
<td>72</td>
<td>71</td>
</tr>
<tr>
<td>Threonine</td>
<td>57</td>
<td>57</td>
<td>59</td>
<td>61</td>
</tr>
<tr>
<td>Serine</td>
<td>121</td>
<td>102</td>
<td>113</td>
<td>106</td>
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<tr>
<td>Glutamic acid</td>
<td>144</td>
<td>149</td>
<td>139</td>
<td>136</td>
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<tr>
<td>Proline</td>
<td>87</td>
<td>95</td>
<td>100</td>
<td>99</td>
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<tr>
<td>Glycine</td>
<td>133</td>
<td>117</td>
<td>116</td>
<td>116</td>
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<tr>
<td>Alanine</td>
<td>66</td>
<td>71</td>
<td>74</td>
<td>76</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>Trace</td>
<td>7</td>
<td>10</td>
<td>10</td>
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<td>Valine</td>
<td>60</td>
<td>70</td>
<td>65</td>
<td>62</td>
</tr>
<tr>
<td>Methionine</td>
<td>1</td>
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<td>5</td>
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<tr>
<td>Isoleucine</td>
<td>39</td>
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<tr>
<td>Leucine</td>
<td>85</td>
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<tr>
<td>Tyrosine</td>
<td>15</td>
<td>18</td>
<td>18</td>
<td>25</td>
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<td>Phenylalanine</td>
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<td>Lysine</td>
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<td>Histidine</td>
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<tr>
<td>Arginine</td>
<td>32</td>
<td>41</td>
<td>50</td>
<td>43</td>
</tr>
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</table>

The concentration. Fig. 5 presents the reciprocal of the sedimentation coefficients of the centers of the two modes of PPC(CP), and of the single mode of PP-L3, as a function of the loading concentration. The zero concentration intercepts correspond to sedimentation coefficients of 14 S for PP-L3, and of 21 and 50 S for the two modes of PPC(CP).

Amino acid analyses of the two disruptive PPC preparations are included in Table III. The two analyses are very similar; the PP-L3 profile is in close agreement with that given by Pal et al. (5). PP-L3 is somewhat enriched in serine and glycine, and relative to PPC(CP) is depleted in lysine, arginine, and the sulfur-containing amino acids.

Experiments were designed to determine the oxidation state of the small amount of cystine in PPC(CP). Fig. 6a depicts g(s) analyses of PPC(CP) immediately before and after incubation at 37° for 2 hours in the presence of reducing agent (10 mM dithiothreitol). A complete loss of the faster sedimenting mode was observed and only one mode, slightly displaced to lower sedimentation coefficients, remained. At the same time, reduction effected a considerable decrease in the viscosity of the preparation, indicating that the changes in centrifugal behavior were manifestations of a disaggregation and not some subtler (i.e. conformational) change. The relative viscosity at 37° of a 1% PPC(CP) solution in 0.15 M KCl-0.05 M Tris-HCl, pH 7.5, dropped from an initial value of 11.4 to a final, stable value of 5.9 within 2 hours after the addition of dithiothreitol to a concentration of 10 mM. PP-L3 showed only a small viscosity change when treated in the same way, as might be expected from the virtual absence of cystine from its amino acid profile. The alterations of sedimentation pattern and of viscosity of PPC(CP) were accompanied by the formation of cysteine sulfhydryl groups reactive with iodoacetamide. Incubation of PPC(CP) in the presence of 40 mM iodoacetamide at room temperature for 16 hours in 0.5 M Tris-HCl, pH 8.5, resulted in no detectable S-
carboxymethylcysteine after acid hydrolysis. Reaction with iodoacetamide under the same conditions after reduction resulted in alkylation of up to 70% of the half-cystine residues.

**PPC from Dissociative Isolations**—The experiments outlined in Fig. 7 were undertaken in order to learn more about the fundamental processes underlying the low shear extraction phenomena described in the first part of this section. Clarified 4 M guanidinium chloride and 3 M MgCl₂ cartilage extracts were examined in the ultracentrifuge, both in the extracting solvents (Fig. 7, a and d) and after dialysis to one-tenth the ionic strength used for extraction (Fig. 7, b and e). In each case, the solute concentration was adjusted to approximately 0.2%. Although solvent density and viscosity variations obviated direct comparison of the results obtained in the high and low ionic strength solvents, the observed changes of sedimentation profiles were so striking that general statements concerning differences in the physical state of PPC in the two types of solvent can be made. In the solvents used for extraction, both magnesium- and guanidinium-extracted solutes gave one predominant peak (labeled II) with a trailing, slowly sedimenting component (labeled III) obscured by the meniscus (Fig. 7, a and d). Mode III was easily visualized after dialysis, and a third, faster sedimenting peak (labeled I) appeared (Fig. 7, b and e); conservation of mass indicated that this new peak was composed primarily of species originally present in Mode II. The relative abundance of Mode I depended on the nature of the extracting solvent: in the magnesium extract, it represented 50 to 60% of the total solute, whereas in the guanidinium extract it accounted for only 20 to 25% of the total.

After the dialyzed extracts were purified by equilibrium density gradient centrifugation as described under "Experimental Procedure," the resulting protein-polysaccharide complexes were investigated in the appropriate low ionic strength solvent (Fig. 7, c and f). The purification step resulted in the loss of Mode III in both cases. A quantitative analysis of the density gradient step for the magnesium-extracted preparation is shown in Fig. 8. All the hexuronic was found at densities greater than 1.73 g per ml, whereas a surface gel containing appreciable amounts of collagen (from amino acid analysis) and a significant amount of soluble 280 nm-absorbing protein and/or glycoprotein were floated to the top of the gradient. Because Mode III does not contain significant amounts of hexuronic, further discussion of it will be limited to pointing out that it may be related to the glycoprotein described by Franek and Dunstone (17). The sedimentation rate of Mode I of guanidinium-extracted solute was decreased significantly by the CsCl purification step (Fig. 7, b and e); no explanation for this alteration can be offered at this time. The very low sedimentation rate of Mode II in 3 M MgCl₂ compared to the other solvents investigated is probably due to the high viscosity of this solvent. It is unlikely that the observed low sedimentation rate is the result of a further disaggre-
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Sedimentation coefficient, s

FIG. 9. Apparent sedimentation coefficient distribution at 20° of reduced and alkylated PPC(Mg) prepared as described under "Experimental Procedure." Solute concentration was 1.5 mg per ml in 0.4 M guanidinium chloride-0.05 M Tris-HCl, pH 7.5. The function g(s) is defined in Equation 1.

The bimodal nature of the g(s) profiles for the purified PPC(Gu) and PPC(Mg) shown in Fig. 7, e and f was reminiscent of the distribution observed for PPC(CP), but important differences were seen. Modes I and II were more distinct, the relative amount of Mode I was greater, especially in PPC(Mg), and the centers of the distributions were shifted toward higher sedimentation coefficients than for PPC(CP). Sedimentation coefficient profiles for two concentrations of PPC(Mg) are shown in Fig. 4c for direct comparison with PPC(CP); Fig. 5 presents plots of the reciprocal of the peak sedimentation coefficients of the two modes of PPC(Mg) as a function of the loading concentration. The centers of the modes for PPC(Mg) in this solvent extrapolate to 25 S and approximately 100 S at zero concentration, values significantly higher than those for PPC(CP).

Reducing agents easily transformed the bimodal distribution of PPC(CP) to a unimodal one after incubation at 37° in 0.5 M CaCl₂ as described above. Under similar conditions, the dissociatively extracted PPC preparations were incompletely disaggregated (Fig. 6, b and c). PPC(Mg) and PPC(Gu) could be completely disaggregated only by conducting the reduction at 70° for 5 min or by reducing and alkylating in the presence of 4 M guanidinium chloride as described under "Experimental Procedure." Pretreating the preparations at 70° did not facilitate later complete disaggregation at 37°. When the reduced dissociatively extracted PPC preparations represented by the dashed lines in Fig. 6, b and c were reacted with iodoacetamide (40 mM at room temperature for 16 hours at pH 8.5), alkylations of 80% of the half-cystine residues in the case of the reduced PPC(Mg) and 87% of the residues in the case of the reduced PPC(Gu) were achieved. Analysis of PPC(Mg) which had been reduced and alkylated in the presence of 4 M guanidinium chloride as described under "Experimental Procedure" indicated complete reduction and alkylation within the limits of the techniques used (±5%); a control alkylation under the same conditions indicated a complete lack of cysteine sulphydryl groups in the untreated PPC within the same limits of uncertainty. The g(s) profile of reduced and alkylated PPC(Gu) is given in Fig. 9. This chemically modified PPC represents 85% of the tissue hexuronate, contains less than 8% protein, and displays a unimodal, symmetrical, and fairly narrow g(s) profile; with its potential for aggregation abolished, it is well suited for measurements of the macromolecular characteristics of undegraded PPC subunits.
In a test of the hypothesis that differences in the profiles of PPC(CP) and PPC(Mg) are the result of shear degradation, a 3 mM MgCl₂ cartilage extract was dialyzed into the Tris-KCl buffer used in the PPC(CP) isolation scheme. Part of the resulting solution was homogenized for 30 min, simulating the disruptive procedure, and part was reserved as a control. Control and homogenized aliquots were then dialyzed into 0.4 mM guanidinium chloride and analyzed in the ultracentrifuge. The calculated g(s) analyses, shown in Fig. 10, indicated that the conditions of homogenization significantly altered the starting distribution. The nature of the alteration is consistent with the hypothesis that shear-induced scission of the macromolecules occurs during disruptive extraction of cartilage. Sonic disruption of a PPC solution for 2 min at 2°C with a Branson Sonifier produced much more impressive fragmentation and sufficed to eliminate Mode I entirely; this is also illustrated in Fig. 10.

The ethanol-salt precipitation and ethanol-ether drying techniques used for isolation of PPC(CP) and PP-L3 also produced small alterations in the sedimentation profile of dissociately extracted PPC; for this reason, we have avoided drying these preparations except for the purpose of obtaining dry weights when needed.

**DISCUSSION**

**g(s) Technique**—Because we have relied heavily on g(s) methodology for evaluating different preparations of proteinpolysaccharides, we will discuss briefly in this section some of the advantages and limitations encountered in using this technique. Some important factors which will affect the apparent g(s) profile when finite solute concentrations are analyzed are (a) boundary spreading due to diffusion, (b) hypersharpening due to concentration dependence of solute sedimentation, (c) the type and concentration of supporting electrolyte, and (d) the Johnston-Ogston effect (14).

Boundary spreading due to diffusion can be superimposed on that due to inherent macromolecular dispersity, and extrapolations of profiles calculated for a number of different times must be made to infinite time in order to correct for it. At a rotor speed of 35,500 rpm, g(s) profiles calculated for PPC(CP) from data obtained at early times and at late times superimposed within experimental error; the contribution of diffusion under these conditions therefore is unimportant to the shape of the g(s) profile.

Thermodynamically meaningful distributions for a particular set of solvent conditions can be made by evaluating data at a number of loading concentrations, correcting for diffusion if present, and extrapolating the curves to zero concentration to obtain an hypothetical ideal g(s) distribution. This ideal distribution is one in which concentration effects due to particle-particle interactions and errors in estimation of the relative amounts of different modes due to the Johnston-Ogston effect are eliminated. No attempts have been made to do these difficult extrapolations because the additional information gained is unnecessary for the purposes of this report. However, the much simpler extrapolations to infinite dilution of the peak sedimentation coefficients of the modes were made and are illustrated in Fig. 5. They provide an unequivocal sedimentation coefficient characteristic of each mode in the chosen solvent and allow comparisons between the different materials. In other experiments, the g(s) method is used for semiquantitative evaluation of differences between various extraction and purification procedures. Care was taken to reproduce when possible the solute concentrations and solvent conditions in order that differences observed in the apparent g(s) profiles can be attributed to real differences in the sedimentation properties of the macromolecules even under nonideal conditions. The use of high supporting electrolyte concentrations, such as 0.5 M CaCl₂, helps minimize the large charge effects exhibited by PPC. Although such a solvent makes the Schlieren optical system difficult to use because of base-line shifts attributable to salt sedimentation, no difficulties were encountered in using interference optics even at very low solute concentrations as long as care was taken to match the air-solvent and air-solution menisci. Guanidinium chloride has a lower density than inorganic salts, and its use as a supporting electrolyte in future experiments may circumvent this problem. Low ionic strength solvents such as 0.1 M KCl are commonly used in centrifugal studies on proteinpolysaccharides (17, 21, 22). Such solvents give much more pronounced hypersharpening of g(s) profiles than the solvents used in the experiments described above, and their use is contraindicated.

Lastly, the g(s) method has one very important consequence even accepting the limitations imposed on it. Fig. 4 graphically illustrates the marked increase in apparent dispersity and sedimentation coefficients exhibited by PPC at concentrations of 1.5 mg per ml and less, well below those often used in centrifugal analyses on related preparations. The boundary spreading exhibited by PPC preparations at high dilutions during sedimentation velocity experiments has been attributed to diffusion. The g(s) analysis indicates that this cannot be the case, and that the broad distributions reflect inherent polydispersity of the preparations. Elucidation of the chemical and physical properties of PPC must take this into account.

**Dissociative Extraction Phenomenon**—The ease with which PPC is mobilized from cartilage at optimal solvent ionic strength is surprising and unprecedented. At concentrations below the optimum for any given electrolyte, extremely long periods of time are needed to extract high yields of the soluble PPC pool without resorting to high shear; Shutton and Schubert (2) found that 90 days of extraction with water at 1°C solubilized only about half the tissue hexosamine. The fact that a critical ionic strength is required before the extraction rate significantly exceeds control levels is suggestive of a polyionic interaction of the PPC, either with itself or with elements of the fibrillar matrix. Interactions of this type, particularly between polyanions and cationic detergents, have been studied extensively by Scott (23); he has found that they are characterized by "critical electrolyte concentrations," below which the polyanion exists as the salt of the cationic detergent and above which it exists as the electrolyte cation salt. Increases in salt concentrations beyond the optimum, on the other hand, decrease the rate of extraction and the ultimate yield of PPC, especially for inorganic electrolytes such as CaCl₂. We have no satisfactory explanation for this phenomenon at this time, but the hypothesis that the decrease in extraction efficiency is the result of a reduced amount of water available for solvation of PPC at very high salt concentrations can be ruled out. Both S-methylisothiooxonium chloride, shown in Fig. 3, and SrCl₂, not shown, extract well in solutions almost saturated with electrolyte. The sharp optima exhibited by inorganic electrolytes indicate that a reasonable amount of care must be exercised in utilizing them for purposes of dissociative extraction. Meyer and Smith (1) used 10% CaCl₂ (approximately 1 M) for extraction of cartilage, whereas Webber and...
Bayley (21) used 2.5 M. Reference to Fig. 1 shows that these two calcium concentrations bracket the optimum and that both fall short of extracting the entire soluble PPC pool. It should be emphasized that all the results presented above were obtained with bovine nasal cartilage and that other cartilages may exhibit different extraction characteristics.

The high extraction efficiency shown by guanidinium and its charged structural analogue S-methylisothiouronium, and the synergistic effect exhibited by urea when used in combination with CaCl₂, suggest that the conformation of the protein moiety of PPC is also necessary for maintaining the integrity of the cartilage matrix. Guanidinium chloride and urea are commonly used protein denaturants, and Gordon and Jeneks (24) have indicated the S-methylisothiouronium is also a good denaturing agent. More convincing evidence of a functional protein conformation in PPC was obtained as the result of reduction and alkylation experiments. Reduction of cystine disulfides and alkylation of the resulting thiol groups with iodoacetamid abolishes the ability of the PPC to form aggregates at low ionic strength. In the two cases investigated, disaggregation of PPC was observed at ionic strengths which were also optimal for its extraction from the tissue. This is taken as firm evidence that aggregation is not a result of intermolecular disulfide bonds and as circumstantial evidence that disaggregation is fundamental to the dissociative extraction process. The evidence in sum indicates that the forces which give rise to aggregation are ionic in nature, and that they are mediated by disulfide-dependent protein conformations which can be destabilized by protein-denaturing agents.

The macroscopic physical appearance of the cartilage slices is not altered by complete dissociative extraction of the soluble PPC pool, even though such extraction removes almost half the dry weight of the tissue. For this reason, we must concur with Hoffman et al. (25) that soluble PPC is not requisite for maintenance of the size and shape of cartilage. Rather it may fulfill the function of a plastic interstitial substance important for absorbing the various stresses the tissue normally experiences. A small residual polysaccharide pool, representing 10 to 15% of the tissue hexuronate, remains behind with the insoluble matrix after efficient extraction by either dissociative or disruptive procedures or by a combination of both methods. This hexuronate pool is completely solubilized by further extraction with base (0.5 M NaOH for 4 hours at room temperature), once more without appreciably altering the physical appearance of the tissue slices. This indicates that the insoluble hexuronate probably exists as chondroitin sulfate chains attached to serine hydroxyl groups in the same manner as in soluble PPC. Pal and Schubert (26) also studied this insoluble pool and found that most of it was solubilized by NH₄OH or KCN under appropriate conditions.

Mobilization of PPC from the tissue may not be the direct result of dissociation of the aggregate observed in soluble PPC. At least two other attractive possibilities exist which are compatible with the evidence presented. Optimal electrolyte conditions may displace PPC macromolecules from a normal association with fixed binding sites located in the insoluble collagen matrix. Such an hypothetical association might be similar to or identical with the one seen for isolated PPC and would help explain why disruptive procedures are required to separate PPC from the matrix at low ionic strength. The insoluble PPC pool remaining in the tissue after extraction could provide the postulated fixed binding sites. Alternatively, dissociative conditions may primarily offset a conformational change in the fibrillar portion of the matrix, such that it becomes more permeable to entrapped PPC species which are coincidentally disaggregated. The fact that disruptively isolated PPC(CP), which has never been exposed to dissociative conditions, is also partially aggregated argues for the existence of some form of PPC aggregate in situ.

Most of the recent studies of PPC have used preparations obtained by disruptive methods. The notable exception is a report by Hoffman et al. (25) who investigated several extraction procedures including one utilizing low shear and low ionic strength at 37°C and pH 5 for 24 hours. These conditions allowed them to isolate tissue polysaccharide as PPC in yields comparable to those obtained after papain digestion of the tissue. Compared to a typical disruptively isolated PPC, this PPC prepared at 37°C was characterized by lower viscosity and lower protein content. For two reasons, we presume that autolysis is the mechanism of extraction at pH 5 and 37°C. First, the presence in cartilage of an enzyme capable of degrading PPC has been reported by Dziewiatkowski, Tourtellotte, and Campo (27); its activity optimum occurs at pH 4, but it shows significant activity at pH 5. Second, the sensitivity of the low ionic extraction to pH and temperature implies an enzymatic process. Control extractions in our studies utilized 0.15 M KCl at room temperature and pH 7.5 for 20 hours and mobilized only 20% of the tissue hexuronate, a value which did not increase significantly during the following 28 hours.

Disruptively Isolated PPC Preparations—Ultracentrifugal evidence indicates that PP-L₃ is a fraction of the slower sedimenting mode of PPC(CP) biased toward lower sedimentation coefficients. Interestingly, it is the faster sedimenting mode of PPC(CP) that is sensitive to reduction at low ionic strength while PP-L₃, which contains only traces of cystine, is not sulfhydryl sensitive. Unfractionated PP-L (4) contains a higher protein content than PPC(CP) but represents a much lower yield of tissue hexuronate. This must reflect the presence in PP-L of the glycoprotein reported by Franek and Dunstone (17) or of other proteinaceous material; PPC(CP) is free of such low density contaminants. The protein content of PPC(CP) is, in fact, not significantly greater than that of the dissociatively extracted protein polysaccharide complexes, a fact which speaks well for the use of cetylpyridinium chloride precipitation techniques for purification purposes. PP-L₃, on the other hand, contains less protein than PPC(CP); this presumably is a reflection of the polydispersity inherent in PPC. PP-L₃ is also highly enriched in galactosamine over glucosamine. The relationship between protein content and glucosamine content of PPC preparations has been documented (5, 28), but a satisfactory explanation for it is lacking at this time.

The question of polydispersity of PPC has been a matter of concern for some time. It may result from shear-induced fragmentation or from enzymatic degradation during extraction. It may also reflect the native state of the macromolecules. The dissociative procedures obviate shear and autolysis, and PPC prepared this way is, in fact, susceptible to depolymerization when subjected to high speed homogenization. For this reason, we cannot recommend the preparation of PPC(CP) to those who are interested in the characteristics of the native state of PPC.
macromolecules, even though the evidence indicates that its method of preparation offers distinct advantages over other disruptive procedures.

**Dissociatively Isolated PPC Preparations**—Sedimentation coefficient profiles of reduced and alkylated PPC(Gu) indicate that this PPC subunit has marked inherent polydispersity which cannot be attributed to degradative isolation artifacts. Although we feel that the distribution in $s$ values implies a significant inhomogeneity in molecular weights, we cannot specify the range represented without additional information about the hydrodynamic properties of the macromolecules. Because of what is known about the specificity of protein synthesis and because PPC is predominantly polysaccharide, we presume that the observed dispersity is primarily caused by a variable number of polysaccharide chains attached to the protein core and/or to a variable polysaccharide chain length. However, other possibilities cannot be ruled out at this time; these include variable core polypeptide chain length and the presence of a multiplicity of discrete core proteins. The inherent polydispersity must reflect anabolic and/or catabolic processes in the cartilage which are involved in maintaining the physiological state of the soluble matrix at the time when the tissue was obtained.

The equilibrium density gradient techniques used in the purification of PPC(Mg) and PPC(Gu) were patterned after those described by Franek and Dunstone (12) and proved to be highly convenient and dependable. The very high densities exhibited by PPC in CaCl$_2$ gradients facilitate the removal of impurities. Furthermore, the high ionic strengths encountered in such gradients should effectively reverse any nonspecific ionic interactions of the polyanion with tissue proteins and/or glycoproteins.

The disulfide-dependent aggregation capabilities exhibited by PPC(Gu) and PPC(Mg) have already been discussed with relation to the potential native state of PPC in the tissue. Two other aspects of the aggregation phenomenon deserve mention at this point. First, the larger amount of aggregate found in PPC(Mg) relative to PPC(Gu) is not reflected in any obvious other aspects of the aggregation phenomenon deserve mention. Second, reduction of PPC(Gu) and PPC(Mg) with dithiothreitol at low ionic strength gives rise to very similar centrifugal profiles which include a small amount of residual aggregate. PPC(CP) treated with reductant under similar conditions is completely disaggregated. Protein disulfides are very commonly found to be resistant to reduction unless the deformation of the protein has been "loosened" by denaturing agents; a discussion of this phenomenon for the case of pepsin is given by Blumenfeld and Perlmann (29). We therefore conclude that the protein moiety of dissociatively extracted PPC, although not necessarily in its native state, is nevertheless more native than that of PPC(CP) because of its resistance to reduction.

The protein portion of PPC has generally been assumed to be a featureless core, the sole function of which was to provide points of attachment for polysaccharide chains. Most recently, Eyring and Yang (30) concluded from optical rotary dispersion and circular dichroism measurements that the protein moiety of PP-L consisted of "highly disordered protein (or proteins) with polysaccharide side chains attached to it." Disulfide and salt linkages between molecules were reported absent. Our findings indicate that at least part of the disorder present in the protein of PPC(CP) and presumably of PP-L is the result of the disruptive isolation scheme used, and that the protein moiety of the native protein-polysaccharide complex of cartilage does in fact possess a conformation which is important for maintaining the integrity of the cartilage matrix.

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