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Protein Polysaccharides from Bovine Nasal Cartilage Interactions with Glycoproteins and the Formation of Aggregates

Vincent C. Hascall Jr

PROTEINPOLYSACCHARIDES FROM BOVINE NASAL CARTILAGE

Interactions with Glycoproteins and the Formation of Aggregates

A thesis submitted to the Faculty of The Rockefeller University
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by

Vincent C. Hascall Jr., B.S.

Approved for publication.
D. Dziewiatkowski
Professor

John D. Gregory, Ph.D.
Associate Professor

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The Rockefeller University
New York, New York

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ABSTRACT

Proteinpolysaccharides are macromolecules which consist primarily of chondroitin-4-sulfate associated with non-collagenous protein. They constitute about half of the dry weight of adult bovine nasal cartilage; the remaining material is predominantly collagen. When cartilage slices are extracted with concentrated salt solutions (typically 4 M guanidinium chloride), 85% of the total proteinpolysaccharide in the tissue is easily solubilized. The collagen framework of the tissue remains intact during the extraction procedure, and the cartilage slices retain their shapes. The amount of proteinpolysaccharide which is solubilized depends, in part, upon the ability of the extraction solvent to dissociate proteinpolysaccharide aggregates which exist in the extracellular matrix of the cartilage. In a *dissociative* solvent, such as 4 M guanidinium chloride, the proteinpolysaccharide in a cartilage extract is disaggregated into subunits. After the extract is dialyzed against an *associative* solvent such as 0.5 M guanidinium chloride, up to 60% of the proteinpolysaccharide molecules reaggregate. The relative amount of aggregate to monomer which is formed depends upon the pH of the extraction solvent; it is a maximum when extraction and aggregation are carried out at pH 5.8.

Procedures which employ equilibrium centrifugation in density gradients were developed to fractionate a cartilage extract. An extract was dialyzed into an associative solvent (0.5 M guanidinium chloride buffered at pH 5.8) in order to aggregate the macromolecules. Cesium chloride was added to a density of 1.69 g/ml, and a gradient was established (40,000 rpm for 44 hours at 20° C in a Spinco SW 50 rotor). The small amount of free glycoprotein and soluble collagen in the dialyzed extract was recovered at the top of the gradient; more than 95% of the proteinpolysaccharide was recovered at the bottom in a fraction referred to as *proteinpolysaccharide complex*, PPC. PPC contained 10% protein, 10% keratan sulfate and 80% chondroitin sulfate. The relative amount of aggregate to subunit in PPC was the same as that in the dialyzed extract. PPC was dialyzed into a dissociative

solvent (4 M guanidinium chloride buffered at pH 5.8) in order to disaggregate the macromolecules. Cesium chloride was added to a density of 1.50 g/ml, and a gradient was established (40,000 rpm for 44 hours at 20° C in a Spinco SW 50 rotor). About 30% of the protein in PPC was recovered at the top of the gradient in a fraction referred to as *glycoprotein link*, GPL; more than 95% of the proteinpolysaccharide was recovered at the bottom in a fraction referred to as *proteoglycan subunit*, PGS. PGS contained 7% protein, 8% keratan sulfate and 85% chondroitin sulfate. In associative solvent conditions, PGS contained no aggregate. PGS reaggregated when it was mixed with GPL. The amount of aggregate regenerated in a mixture was greater when the relative amount of GPL to PGS was greater.

The non-covalent interactions between PGS and GPL that are required for aggregation are mediated by conformations of the protein moieties within the macromolecules. Aggregation is reversed by solvent pH below 4, by high ionic strengths such as 2 M guanidinium chloride or 3 M $MgCl_2$, and by protein denaturants such as 1% sodium dodecylsulfate or 6 M urea. Aggregation is irreversibly abolished when cystine residues within the proteins are reduced. Although sulfhydryl reducing agents and low solvent pH prevent aggregation, they are not sufficient conditions to allow GPL to be separated from PGS. This suggests that aggregation requires at least two distinct interaction sites between GPL and PGS.

Viscosimetric and centrifugal studies indicate that the PGS molecules have large effective hydrodynamic volumes (28-56 ml/g in 0.5 M guanidinium chloride), and little, if any, asymmetry (axial ratios of less than 4). The average number of PGS molecules per aggregate is about 13. The PGS fraction is polydisperse; 80% of the macromolecules have molecular weights between 1.3 and 4.1 million. This polydispersity in molecular weights is due primarily to differences in the amount of chondroitin sulfate attached to the protein moieties of the PGS macromolecules.

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CHAPTER I

GENERAL INTRODUCTION

A. Macromolecular structure of cartilage

Nasal septum is an example of the class of connective tissues known as hyaline cartilages. These cartilages combine strength and rigidity with a great degree of resilience. Such properties are important for the structural functions of these tissues in an organism; and they are, to a large extent, the result of the tissue's macromolecular composition and organization. Hyaline cartilage consists of a relatively small population of chondrocytes which occupy lacunae within an extensive extracellular matrix. The extracellular matrix is constructed primarily from two types of macromolecules, collagen and the proteinpolysaccharides.* In adult nasal cartilage these two types of macromolecules are present in roughly equal amounts and account for more than 90% of the tissue dry weight. The collagen portion of the extracellular matrix is organized into a network of interlaced fibers. The remaining non-fibrous portion of the extracellular matrix is referred to as the amorphous ground substance of the tissue, and it is embedded between and around the fibrous collagen network. Proteinpolysaccharides constitute all but a few percent of the ground substance; they consist of polysaccharide chains, mainly chondroitin-4-sulfate, which are covalently attached to non-collagenous proteins. In solution, they are highly hydrated, and much of the 70% water content of the tissue is probably associated with these macromolecules. The tissue, then, can be thought of as a very viscous semi-gel of proteinpolysaccharide macromolecules which surround an interlaced network of collagen fibers.

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* The term *proteinpolysaccharide* was originally proposed by Gerber, Franklin and Schubert, 1960, to describe particular preparations from bovine nasal cartilage which were "composed of protein and polysaccharide." Since then, however, the term has become a generic name for all preparations obtained from hyaline cartilages which consist primarily of chondroitin-4-sulfate associated with non-collagenous protein. It is used in this thesis in this broader, more general sense.

Some of these features of the extracellular cartilage matrix are seen in the electron micrographs shown in Figure 1, which were kindly provided by Gretchen Hascall. The picture on the left shows a portion of the interterritorial matrix of bovine nasal cartilage.* The fibrous part of the matrix is not readily visible, although its presence is indicated by the shadowy lines in the picture. The most prominent feature of the micrograph is the abundance of densely stained matrix granules. These matrix granules contain proteinpolysaccharide (Matukas, Panner and Orbison, 1967). They are most likely precipitation products of the lead or uranyl salts of proteinpolysaccharides. In their native state in the tissue, the proteinpolysaccharides are hydrated and may have occupied the large unstained areas seen in the picture. The micrograph on the right shows the interterritorial matrix of nasal cartilage after the ground substance material has been extracted with 4 M guanidinium chloride, a procedure that will be described in chapter II. The collagen fibers are now much more prominent. They appear to be more or less randomly oriented; some fibers can be found aligned in every direction, and some are seen in cross section. The overall impression is that the fibrous matrix is composed of a felted arrangement of long collagen fibers.

The two micrographs suggest how closely the ground substance and fibrous portions of the matrix are associated. The collagen fibers provide a structural framework and shape for the tissue; but by themselves, they lack the rigidity and the resistance to deformation which are attributes of the intact cartilage (Sajdera, 1969). The composite structure of fibrous matrix plus ground substance, then, has physical properties which are different from either component of the matrix by itself, but which result from the macromolecular arrangement of the two.

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* The matrix of hyaline cartilage consists of two distinct regions, territorial matrix which immediately surrounds the chondrocytes, and interterritorial matrix which constitutes the rest. The two appear to be composed of the same types of macromolecules but are apparently organized in different ways. They can often be distinguished histochemically.

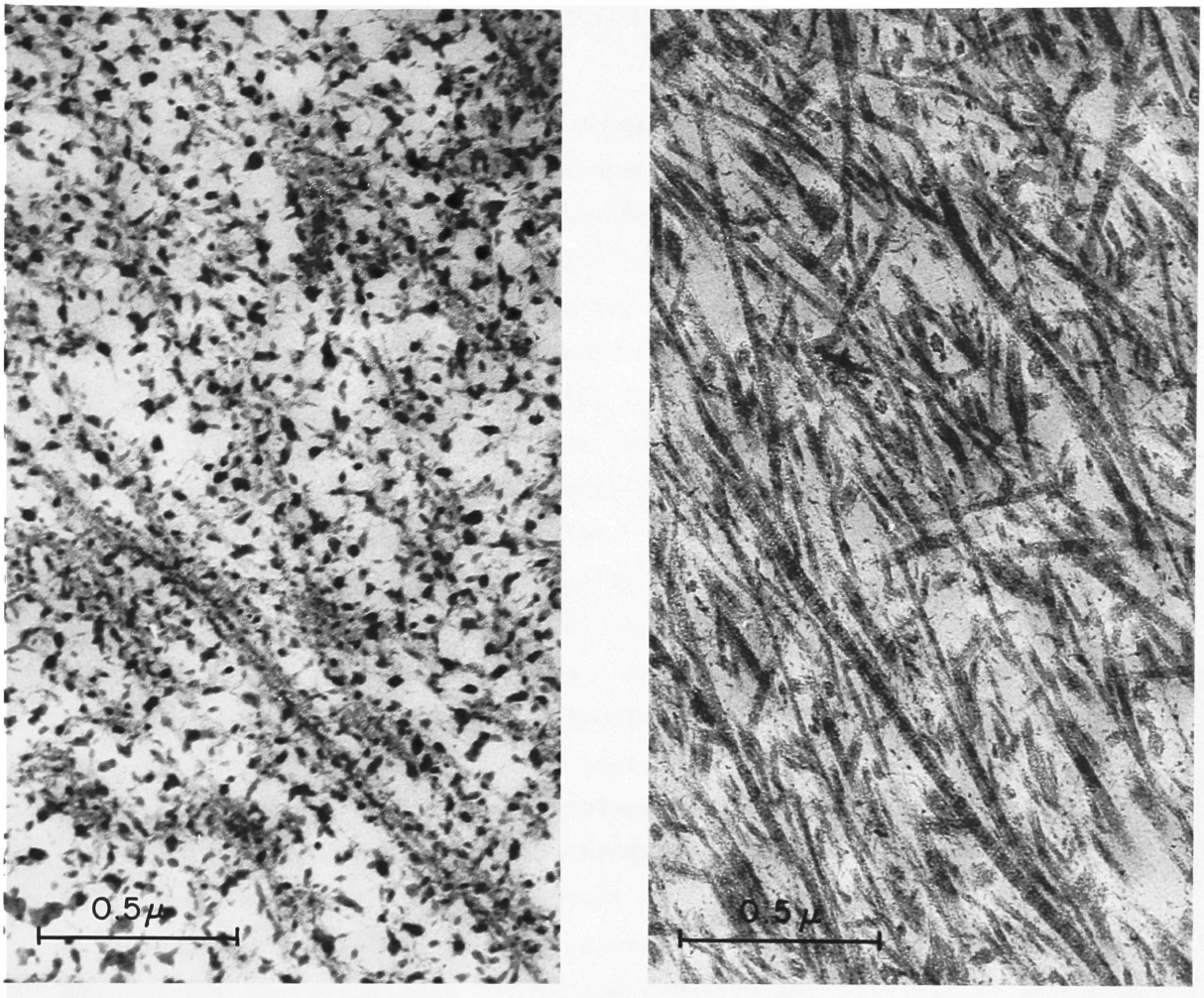

Figure 1. Electron micrographs of interterritorial matrix of bovine nasal cartilage. Samples of cartilage were fixed in 2% glutaraldehyde in buffered phosphate at pH 6.7 either directly (left) or after extraction of the ground substance material from the tissue with 4 M guanidinium chloride (right). The fixed samples were post-fixed with 1% OsO_4 and were embedded in Epon. Ultrathin sections were made and were stained with 2% uranyl acetate and 0.3% basic lead citrate.

The samples were prepared, and the sections observed in the electron microscope by Gretchen Hascall.

B. Polysaccharides of cartilage

Nasal cartilage contains two polysaccharides, chondroitin-4-sulfate and keratan sulfate. These polysaccharides are composed of unbranched sequences of the repeating disaccharides indicated in Figure 2; the repeating distance of a disaccharide unit is about 8-10 Angstroms. The chemical details of the structures for the repeating disaccharides were determined primarily through the work of Wolfrom, Madison and Cron, 1952, and Wolfrom and Juliano, 1960, for chondroitin sulfate; and through the work of Hirano, Hoffman and Meyer, 1961, and Rosen, Hoffman and Meyer, 1960, for keratan sulfate. A recent review of this work is found in Brimacombe and Webber, 1964. Both chondroitin sulfate and keratan sulfate have sulfate esters in their structures which carry negative charges in solution at all pH values that are practical for studying the intact macromolecules. In addition to the sulfate ester on the galactosamine residue, chondroitin sulfate has a free carboxylate group on the glucuronic acid residue. The carboxyl group is thought to be fully ionized in solutions with pH values greater than 5 (Warner and Schubert, 1958), and chondroitin sulfate consequently contains two negative charges per repeat unit at physiological pH. The high charge densities of the polyanionic polysaccharides are responsible for the characteristic basophilia observed in histological sections from cartilages.

The chondroitin sulfate chains in bovine nasal cartilage are generally thought to have 30 to 50 dissacharide units for molecular weights of 15-25,000 (Partridge, Davis and Adair, 1961, Buddecke, Kröz and Lanka, 1963, Anderson, Hoffman and Meyer, 1965, Luscombe and Phelps, 1967b) although a lower value, 14,000, has been determined for chondroitin sulfate chains from porcine nasal cartilage (Marler and Davidson, 1965). Higher values, between 40-50,000, have been suggested (Mathews and Lozaityte, 1958), but these estimates apparently were made on chondroitin sulfate preparations which contained doublet structures, two individual chondroitin sulfate chains bound together by a short polypeptide

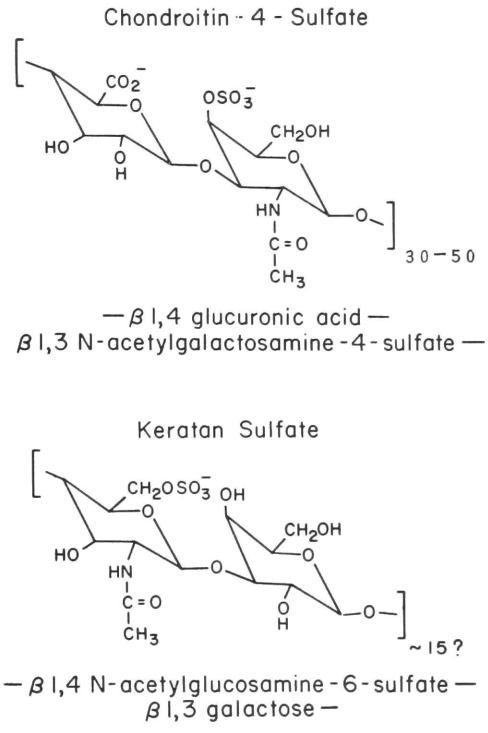

Figure 2. Chemical structures of the repeating disaccharide units of chondroitin-4-sulfate and keratan sulfate.

sequence (Luscombe and Phelps, 1967b, Mathews, 1968a). The size of the keratan sulfate chains in nasal cartilage is uncertain; in other tissues this polysaccharide exists as short chains with 10-25 disaccharide units terminated at the non-reducing end by a sialic acid residue (page 59, Schubert and Hamerman, 1968). Because these two polysaccharides are the only ones known to be present in nasal cartilage, the amounts of each in samples isolated from the tissue can be estimated from the amount of glucuronic acid or galactosamine (for chondroitin sulfate) and glucosamine (for keratan sulfate) contained in the samples. Galactose is present in other saccharide structures in the tissue and cannot be used as an indication of keratan sulfate alone (see section D below).

C. Current model for proteinpolysaccharide structure

Shatton and Schubert, 1954, were the first to present evidence that, in nasal cartilage, chondroitin sulfate exists firmly attached to non-collagenous protein. Mathews and Lozaityte, 1958, subsequently presented data which suggested that the structure of a proteinpolysaccharide macromolecule consisted of a protein core with a number of chondroitin sulfate chains attached to it. Results described by Partridge, Davis and Adair, 1961, supported this model; and, on the basis of reduction studies, these authors proposed that the chondroitin sulfate chains were unbranched and had molecular weights around 28,000. Their evidence seemed to indicate that the reducing termini of the polysaccharide chains were free. Partridge and Elsden, 1961, treated proteinpolysaccharide with dilute base which breaks the bond between protein and chondroitin sulfate (see section D below). They then separated the protein fraction from chondroitin sulfate using ion exchange chromatography. Because glucosamine, one of the saccharides of the repeating unit of keratan sulfate, remained bound to the protein fraction, these authors suggested that proteinpolysaccharide macromolecules contained keratan sulfate. Gregory and Rodén, 1961, provided evidence for this suggestion when they isolated keratan sulfate from bovine nasal proteinpolysaccharide which had been extensively digested with hyaluronidase and proteolytic enzymes. Although subsequent work

has shown that the reducing ends of the chondroitin sulfate chains are not free, the basic model for proteinpolysaccharide structure proposed by Mathews and Lozaityte as modified by Partridge and co-workers is currently accepted. The proteinpolysaccharides consist of 8-20% protein, 10% keratan sulfate and the remainder chondroitin-4-sulfate. All constituents are thought to be covalently bound into one macromolecular species with the protein presumably functioning as a core to which polysaccharide chains are attached.

D. Attachment sites between polysaccharide and protein

Muir, 1958, first suggested that serine residues were involved in the attachment of chondroitin sulfate to protein in proteinpolysaccharides. She observed that half of the original serine content of a proteinpolysaccharide preparation from porcine tracheal cartilage remained attached to chondroitin sulfate after extensive proteolytic digestion with papain, whereas other amino acids were, for the most part, removed. The amount of serine left was about one residue for a chondroitin sulfate chain molecular weight of 25,000. Serine was subsequently shown to be the site of attachment by two groups of investigators; Rodén and co-workers (Gregory, Laurent and Rodén, 1964, Rodén and Smith, 1966, Rodén, 1968) and Anderson, Hoffman and Meyer, 1965. The former workers used hyaluronidase and proteolytic enzymes on a proteinpolysaccharide preparation from nasal cartilage and isolated a fraction which consisted of small peptides with oligosaccharides attached. Structural work on this fraction indicated that the attachment between protein and chondroitin sulfate consisted of a glycosidic bond between the hydroxyl of a serine within the protein and a xylose residue at the reducing end of the polysaccharide chain, Figure 3. Two galactose residues are then attached to the xylose through the β-glycosidic bonds indicated. The second galactose is then attached to the repeating sequence of chondroitin sulfate through a β 1-3 bond with glucuronic acid. The data for this structure are summarized by Rodén, 1968.

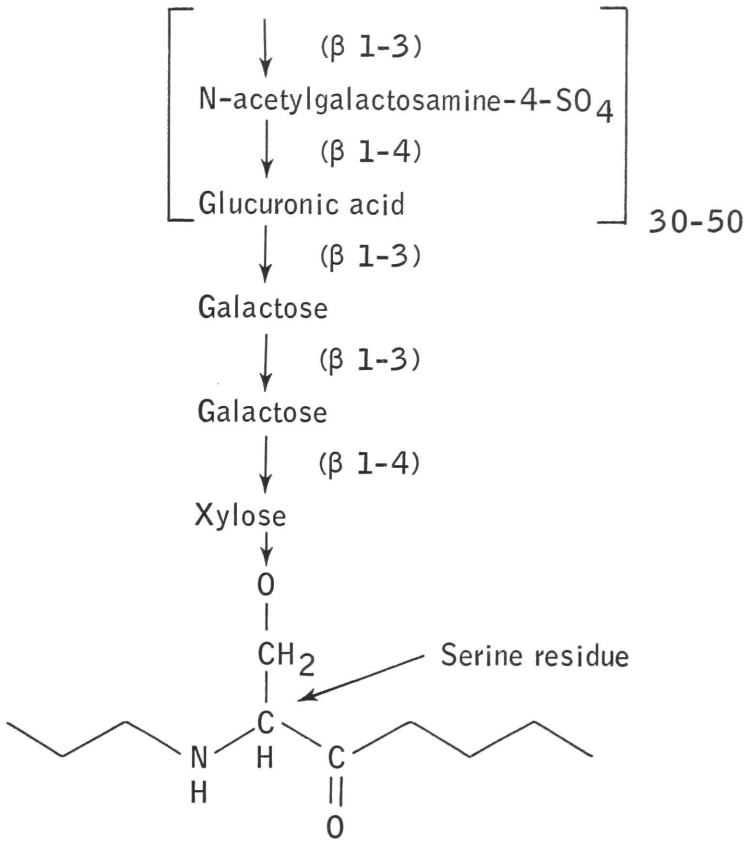

Figure 3. Structure of the attachment region between protein and chondroitin sulfate in proteinpolysaccharides.

Anderson *et. al.*, 1965, used different methods to deduce that serines in the polypeptide were involved in the attachment region between chondroitin sulfate and protein. They observed a loss of serine when proteinpolysaccharides were treated with 0.5 M NaOH at room temperature for 19 hours. They subsequently demonstrated that this loss was the result of the β-elimination mechanism pictured in Figure 4. Base catalyzes the reaction by removing the alpha hydrogen from the serine; the chondroitin sulfate chain is readily eliminated with the formation of a dehydroalanine residue in the polypeptide. The dehydroalanine intermediate is converted to pyruvic acid in the acidic conditions of the hydrolysis step for amino acid analysis. Unsubstituted serines are not affected by the base treatment, and ideally one residue of serine is lost for each chondroitin sulfate chain that is eliminated. The same authors presented strong evidence for the elimination mechanism by reducing the dehydroalanine residues before acid hydrolysis and subsequently observing an increase in the alanine content in the amino acid analyses; this is also indicated in Figure 4. Their experiments provided a chemical mechanism for explaining one of the oldest observations known for the properties of cartilages; namely, that extraction of cartilage with mild base readily solubilizes most of the tissue chondroitin sulfate (Morochowetz, 1877, and Krukenberg, 1884). A few experiments are described in chapter IV which attempt to use this β-elimination reaction to determine the number average molecular weights of chondroitin sulfate chains from different proteinpolysaccharide fractions.

The manner in which keratan sulfate is attached to the protein in nasal cartilage proteinpolysaccharides is unknown. The linkage is apparently stabile to treatment with 0.5 M NaOH for 20 hours at room temperature (Partridge and Elsden, 1961). This makes it unlikely that the linkage is either serine or threonine as has been suggested for keratan sulfate which was isolated from human rib cartilage and shark cartilage (Seno, Meyer, Anderson and Hoffman, 1965) and human tracheal cartilage (Castellani, 1968). The linkage also appears to be stabile to

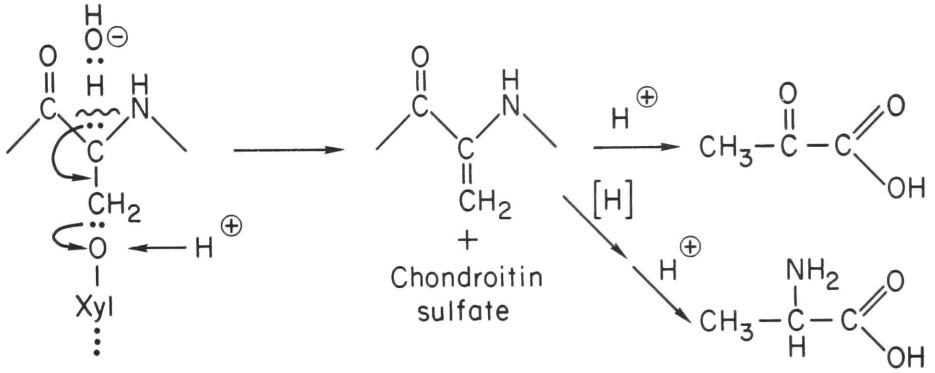

Figure 4. Base catalyzed β-elimination of chondroitin sulfate chains from serine hydroxyl groups. The hydroxyl of the serine, which is drawn at the left as part of a polypeptide, is attached through a glycosidic bond with a xylose at the reducing end of a chondroitin sulfate chain. The hydroxide ion, in mild basic conditions, catalyzes the elimination of the chain from the β-carbon of serine by removing the α-hydrogen ion; the electrons shift as indicated to produce a dehydroalanine residue in the polypeptide and a free chondroitin sulfate chain. The dehydroalanine is either converted directly to pyruvate (upper right) by acid hydrolysis or to alanine (lower right) by reduction before the hydrolysis step. Unsubstituted serines in the polypeptide are unreactive to the β-elimination mechanism.

treatment at room temperature with 1 M hydroxylamine, pH 7.4 (B. Fulpius, unpublished observations), which makes it unlikely that it is an ester linkage. It may be an asparaginyl-glycosidic bond as has been suggested for keratan sulfate from cornea (Seno *et. al.*, 1965, Castellani, 1968).

E. Objective of the present work

At present, in spite of a great deal of thoughtful and diligent work in a number of laboratories, there is little agreement as to what the hydrodynamic characteristics such as the sizes, shapes or molecular weights of the proteinpolysaccharides are. In fact, there is an uncertainty about how many different types of proteinpolysaccharides exist in the tissue. The macromolecules are firmly bound in the cartilage matrix, and disruption of the tissue by high-shear homogenization is generally required in order to recover more than 30% of its proteinpolysaccharide content. The final products isolated after such an extraction step depend upon subsequent purification and fractionation procedures. Such preparations can represent from 25-80% of the tissue proteinpolysaccharide and can contain from 8-25% protein. Investigators have tacitly assumed that the properties of such preparations reflect those of the macromolecules in their native states, although it has been recognized that the vigorous isolation procedures may introduce some shear degradation of the macromolecules (page 73, Schubert and Hamerman, 1968).

Recently, Sajdera and Hascall, 1969, described new methods for extracting proteinpolysaccharide from bovine nasal cartilage. They found that particular concentrations of different salt solutions, such as 2 M $CaCl_2$, 3 M $MgCl_2$ or 4 M guanidinium chloride, allowed 85% of the proteinpolysaccharide in cartilage slices to be solubilized without the necessity for tissue homogenization. The procedures were termed *dissociative* because evidence indicated that efficient extraction of proteinpolysaccharide from the cartilage depended upon the ability of the extracting medium to dissociate proteinpolysaccharide aggregates in the tissue matrix. *In vitro*, proteinpolysaccharide preparations isolated by dissociative methods exhibited a reversible aggregation under appropriate

solvent conditions. In addition, a comparison of proteinpolysaccharide prepared by dissociative extraction with proteinpolysaccharide prepared by a method which utilized high speed homogenization of the tissue indicated that the latter method significantly shear degraded the macromolecules. These results suggested that it would be useful to determine in more detail the causes and properties of proteinpolysaccharide aggregation, and that some of the physical characteristics of proteinpolysaccharides isolated by dissociative extraction procedures should be reinvestigated. Experiments were designed to explore these two major areas, and they are described in the remainder of this thesis.

The next chapter discusses data which suggest that aggregation requires interactions between two distinct types of macromolecules, small glycoproteins and much larger proteinpolysaccharides. The third chapter describes some of the chemical characteristics of these interactions which are required for aggregation. The fourth chapter describes physical measurements of the subunit and aggregate forms of proteinpolysaccharides. The final chapter briefly summarizes the results and proposes a model for proteinpolysaccharides which is consistent with the results presented in this thesis.

CHAPTER II

THE FUNCTION OF GLYCOPROTEIN IN THE FORMATION OF PROTEINPOLYSACCHARIDE AGGREGATES

A. Introduction

Shatton and Schubert, 1954, were the first to report the actual isolation of a proteinpolysaccharide from hyaline cartilage. They described a preparation from bovine nasal cartilage which consisted of chondroitin sulfate and non-collagenous protein and suggested that the protein and polysaccharide were covalently combined. In their procedures, tissue was extracted with water at 4°C for periods of several days. Even when extraction was continued for 90 days, only about 50% of the total proteinpolysaccharide in the tissue was solubilized. Later, Malawista and Schubert, 1958, reported methods for increasing the yields of soluble proteinpolysaccharide to about 80% of the total. They homogenized dried cartilage into water with a rotory homogenizer operated at high speeds; proteinpolysaccharide was isolated from the clarified homogenate. The fact that long extraction times or high-shear, mechanical disruption of the cartilage was required to extract proteinpolysaccharide suggested that the macromolecules were tightly bound into the structure of the tissue.

Gerber, Franklin and Schubert, 1960, subsequently devised a centrifugal method for fractionating soluble proteinpolysaccharide into two fractions on the basis of sedimentation characteristics. The fractions were called *proteinpolysaccharide light*, PP-L, and *proteinpolysaccharide heavy*, PP-H. Pal, Doganges and Schubert, 1966, developed a more complex differential sedimentation method which separated PP-L into four fractions, designated PP-L3, PP-L4, PP-L5 and PP-L6. The work of Schubert and co-workers has been carried out with care and insight, and it has greatly influenced the direction of thought and research by workers in this field. Nevertheless, the isolation techniques developed by these workers have not led to a clear understanding of the structure of proteinpolysaccharides or of their role in the macromolecular organization of the cartilage.

Sajdera and Hascall, 1969, discovered that certain concentrations of different electrolytes facilitated solubilization of proteinpolysaccharide

from cartilage slices without the necessity for tissue homogenization. A method was described for extracting and purifying 85% of the total tissue proteinpolysaccharide. Because the preparation encompassed all of the fractions described by Schubert and co-workers, it was called *proteinpolysaccharide complex*, PPC. Under certain solvent conditions, PPC exhibited a reversible aggregation; in *dissociative* solvents such as 4 M guanidinium chloride only one component was observed in the ultracentrifuge, while in *associative* solvents such as 0.5 M guanidinium chloride a second, faster sedimenting peak appeared. Because electrolyte concentrations which dissociated PPC were also efficient in extracting proteinpolysaccharide from the cartilage, it was proposed that proteinpolysaccharides existed in the ground substance of the cartilage to a great extent as aggregate complexes which were entrapped in the fibrous matrix. When these aggregates were dissociated into subunits, the subunits were able to diffuse out of the tissue. This hypothesis offered an explanation for why proteinpolysaccharides are firmly embedded in the cartilage and why homogenization is required to extract them in low ionic strength solvents.

Because an understanding of the mechanisms involved in aggregation of PPC *in vitro* might suggest models of how the ground substance *in vivo* is organized, the aggregation process was studied in more detail. Some of the experiments from this investigation are described in this chapter. Evidence is presented which shows that aggregation of proteinpolysaccharide results from non-covalent interactions between two different types of macromolecules. The first, which will be referred to as *proteoglycan** *subunit*, PGS, contains more than 95% of the weight of PPC and is

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* The term *proteoglycan* is used to describe macromolecules which are primarily polysaccharide. The polysaccharide is postulated to be covalently bound to the small amount of protein present. The term is introduced to avoid the confusion with the PP-L nomenclature of Schubert and co-workers which would result if this fraction were referred to as proteinpolysaccharide subunit.

predominantly polysaccharide. The second, which will be referred to as *glycoprotein link*, GPL, contains less than 3% of the weight of PPC and is primarily protein. PGS aggregates in the presence of GPL, and the amount of aggregate in mixtures is greater when the proportion of GPL to PGS is greater.

B. Experimental procedure

1. Materials

Guanidinium chloride (ultrapure) was purchased from Mann Research Laboratories. Cesium chloride (99.95%) was obtained from A. D. Mackay, Inc. 2-(N-morpholino)ethanesulfonic acid monohydrate, A grade, subsequently abbreviated as MES, was obtained from Calbiochem. All other chemicals were reagent grade.

2. Analytical methods

a. Hexuronic acid -- Glucuronic acid is the only known hexuronic acid in bovine nasal cartilage. Also, glucuronic acid accounts for about 25% of proteinpolysaccharide preparations.* For these reasons, the relatively easy and specific carbazole method for measuring hexuronic acid in samples was used in order to estimate the approximate amount of proteinpolysaccharide present in the cartilage fractions described in this thesis. In all experiments, hexuronic acid was determined by the modification of Bitter and Muir, 1962, of the carbazole method of Dische, 1947. In extraction experiments, some proteinpolysaccharide remained bound in the tissue. In order to determine the amount of hexuronic acid in this insoluble fraction, the extracted tissue was dispersed by heating at 100°C in about 10 volumes of 3 N sulfuric acid for 30 minutes. Control experiments with soluble proteinpolysaccharide showed no detectable loss of hexuronic acid as a result of this treatment.

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* See section 5 of Results and Discussion, this chapter.

b. Hexosamines -- Most of the samples described in this chapter were in solutions which contained guanidinium chloride. Since the chromatographic systems which were used to measure the amounts of hexosamines or of amino acids in samples are sensitive to the presence of guanidinium chloride, samples which were to be hydrolyzed for hexosamine analysis were first dialyzed against 0.1 M KCl. After dialysis, the hexuronic acid concentrations of the samples were determined, and then measured aliquots were hydrolyzed in 4 N HCl at 100°C in evacuated, sealed tubes for 8 hours. The samples were flash dried and dissolved in water. The amounts of glucosamine and galactosamine in the hydrolysates were then determined on the Beckman amino acid analyzer. The chromatographic system of Spackman, Stein and Moore, 1958, for measuring basic amino acids was used, but a longer resin column (17.5 x 0.9 cm) than that normally used for amino acids (10 x 0.9 cm) was required to achieve better resolution of the hexosamines. The amounts of glucosamine and galactosamine per glucuronic acid in the samples were then calculated.

c. Amino acids -- Samples were first dialyzed against 0.1 M KCl and then their glucuronic acid concentrations were determined. Measured aliquots were hydrolyzed in 6 N HCl at 110°C in evacuated, sealed tubes for 20 hours. The samples were then flash dried and dissolved in water. Amino acids were then measured on the Beckman amino acid analyzer by the method of Spackman, Stein, and Moore, 1958. The amino acid contents in residues per 1000 residues were calculated. The total amino acid recovery per glucuronic acid content in a sample provided an estimate of its relative amount of non-dialyzable polypeptide.

d. Hexose -- Total hexose contents of samples were determined by the anthrone method described by Yemm and Willis, 1954; galactose was used as a standard. Corrections for the hexuronic acid and hexosamine content of the samples were estimated from controls as described by Doganges and Schubert, 1964.

e. Sialic acid -- The sialic acid contents of samples were measured by the thiobarbituric acid method described by Aminoff, 1961.

3. Physical methods

a. Analytical ultracentrifugation -- All 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 35,500 rpm. Dilute proteinpolysaccharide solutions (0.25% w/v or less) were investigated in cells equipped with 30 mm Epon double-sectored centerpieces and sapphire windows. The camera lens was focused two-thirds of the height of the solution column above the lower window. Interference patterns of the boundary were photographed on Kodak type II-G plates at various times after an experiment was initiated. Fringe resolution across the boundary was usually apparent within an hour under these conditions. A Gaertner comparator was subsequently used to measure the fringe displacements on the photographic plates as a function of radial distance. The data were then transferred to IBM cards and read into a Control Data 160G computer which was programmed to calculate g(s) distribution functions of the solute sedimentation coefficients. The g(s) function is defined as follows:

$$g(s) = (1/c_0)(dc/ds) \qquad (1)$$

where c_0 is the loading concentration, c is the concentration at a given radial position and time, and s is the sedimentation coefficient associated with that radial position and time. In terms of the actual parameters measured, the function has the following form:

$$g(s) = (1/n_0)\omega^2 t(r^2/r_m^2)r(dn/dr) \qquad (2)$$

where ω is the angular velocity of the rotor in radians per second, t is the time in seconds that the centrifugal field was applied to the sample (corrected for the acceleration time), r is the radial distance in centimeters, r_m is the radial position of the air-solution meniscus, the ratio (r^2/r_m^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 due to the solute. The methods used for the calculation were essentially those of Schumaker and Schachman,

1957, but, whereas they determined $\Delta c/\Delta r$ from graphs of concentration with respect to radial distance, computer methodology was used in these experiments to evaluate dn/dr by differentiation of second order least squares polynomials fitted to groups of 5 to 11 (n,r) points across the solute boundary. A theoretical treatment of this method is given by Williams, VanHolde, Baldwin and Fujita, 1958; the first application to polydisperse polysaccharide systems was by Williams and Saunders, 1954. The g(s) function provides a quantitative method for measuring the extent of polydispersity in the sedimentation velocities of the macromolecules under the particular solvent and centrifugal conditions of the experiment. It allows different experiments to be compared directly; and, as experiments in chapter IV show, the technique can be used to estimate the range of molecular weights for proteinpolysaccharides. A more detailed presentation of the method for calculating the distribution functions, and some discussion of the advantages and limitations of the technique for evaluating centrifugal data for proteinpolysaccharides are presented in the Appendix.

b. Viscometry -- The viscosities of proteinpolysaccharide solutions were measured with an Ubbelohde dilution viscometer equipped with four bulbs in order to provide four different shear stresses. The viscometer was obtained from Cannon Instruments Company (State College, Pa.) and is designated by them as type 100; the shear stress range characteristic of this instrument for solution densities near 1 g/ml is 1.9 to 18.7 dynes/cm^2. Viscosity measurements were made at 25.0°C on solutions which had been pressure-filtered directly into the viscometer through coarse grade sintered glass to remove dust particles. The constants used to calculate the rates of shear were those supplied with the viscometer. The shear stresses were determined experimentally with 0.5 M guanidinium chloride, the solvent used for most of the studies reported below. The viscosity of 0.5 M guanidinium chloride, η_0, was calculated to be 1.017 centistokes from equation 5 of Kawahara and Tanford, 1966.

The procedure used for calculating the viscosity of a solution, η, under the ideal conditions of zero rate of shear is described in detail by Sajdera, 1969. An outline of the method is as follows. The viscosity of a Newtonian fluid can be defined as the rate of change of an applied shear stress, τ, which is required to produce a given change in the rate of shear, G.

$$\eta = d\tau/dG \qquad (3)$$

This equation is true for non-Newtonian fluids only in the limit of zero rate of shear, $\tau = 0$. For each solution, then, the measured values of the rate of shear were corrected for non-Newtonian flow as suggested by Yang, 1961, (equation 47, page 376). These corrected values and the point $G,\tau = 0,0$ were then fit by the methods of least squares to a polynomial of the defined shear stress values.

$$G(\tau) = k_0 + k_1\tau + k_2\tau^2 + k_3\tau^3 \qquad (4)$$

where the k_i are the polynomial coefficients. Closeness of the fit of the polynomials was evaluated by analysis of variance and by observation of the magnitude of k_0, which must be zero for equation 4 to be valid; k_0 never exceeded the standard deviation of the G points from the calculated polynomial. The value $dG/d\tau$ at $\tau = 0$ is the first order coefficient for the polynomial, k_1, and from equation 3 at $\tau = 0$, the reciprocal of k_1 is an estimate of the solution viscosity.

$$\eta = 1/k_1 \qquad (5)$$

The data in this chapter are presented as the specific viscosity, η_{sp}.

$$\eta_{sp} = (\eta - \eta_0)/\eta_0 \qquad (6)$$

4. Fractionation of cartilage extract

a. Preparation of tissue -- Nasal septa from 1- to 2-year-old cattle were obtained from an abattoir within 1 hour of slaughter, were freed of adhering non-cartilaginous tissue and perichondrium, and were rinsed with isotonic saline solution at 4°C. The septa were then sliced with a Stanley Surform, a woodworking tool that can be purchased at many hardware stores. It functions in a manner similar to a kitchen

grater and facilitates the preparation of thin, uniform slices of cartilage. Slices prepared in this way were about 0.5 mm in thickness and were stored in sealed containers at -15°C until used.

b. Preparation of cartilage extract -- Approximately one gram of nasal cartilage slices per 15 volumes of 4 M guanidinium chloride, 0.05 M 2-(N-morpholino)ethanesulfonic acid, MES, or 0.05 M sodium acetate, pH 5.8* was stirred at room temperature by means of a magnetic stirring bar. For preparative purposes, the extraction solution was freed of slices and particulate matter after 24 hours 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, which will be referred to as *cartilage extract*, contained approximately 0.6% (w/v) proteinpolysaccharide.

In one experiment, the solubilization of proteinpolysaccharide from the tissue was measured as a function of extraction time in two solvents; the 4 M guanidinium chloride solvent just described and 0.15 M KCl, 0.05 M tris-HCl, pH 7.5. The data were tabulated as the percentage of the total hexuronic acid in the slices released into the solvents at different times after the beginning of the experiment

c. Preparation of proteinpolysaccharide complex, PPC -- The cartilage extract was dialyzed against 7 volumes of 0.05 M MES, pH 5.8 for 16 hours at room temperature; this brought the retentate to 0.5 M in guanidinium chloride. Modifications of the density gradient methods described by Franek and Dunstone, 1966, and by Franek and Dunstone, 1967, were then used to remove soluble collagen and other low-density proteins and glycoproteins from proteinpolysaccharide. Solid CsCl was added to the dialyzed cartilage extract to give a solution density of

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* The pH value of the extraction solvent was selected to maximize the yield of aggregate recovered in later steps; this is discussed in chapter III.

1.69 g/ml (1.19 g of CsCl per g of solution). The solution was centrifuged at 40,000 rpm in a Spinco SW 50.1 rotor for 44 hours at 20°C in order to establish an equilibrium density gradient (density gradient 1). The tubes from the gradient were sliced into 5 fractions with a Spinco tube slicer. A thin film of collagen which collected at the top of the gradient was removed by filtering the top fraction through a glass wool plug. The densities of the fractions were measured by using a 500 microliter pipette as a pycnometer. The fractions were then dialyzed against 0.5 M guanidinium chloride to remove CsCl, and the absorbance at 280 mμ and the hexuronic acid content of each were measured. The results of such an analysis are shown in the top half of Figure 5. Most of the hexuronic acid and about 60% of the absorbance at 280 mμ contained in the cartilage extract was recovered in the bottom two fractions of the gradient*; the material in these fractions will be referred to as *proteinpolysaccharide complex*, PPC. The top fraction contained most of the remaining soluble material in the cartilage extract which had a significant amount of absorbance at 280 mμ. Analytical data presented later indicate that the material in this fraction is primarily protein, and it will be referred to as *glycoprotein-I*, GP-I. The second and third fractions probably contain small amounts of material from both the GP-I and PPC fractions; for analytical purposes they were discarded.

For preparation of larger quantities of PPC, the Spinco type 40 angle rotor was used. Centrifugation was carried out at 34,000 rpm for 48 hours at 20°C; and PPC was recovered in the bottom 2/5 of the gradient.

d. Preparation of proteoglycan subunit, PGS, and glycoprotein link, GPL -- Two methods were developed to isolate proteoglycan subunit. The first uses PPC as a starting material and allows recovery of glycoprotein

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* In many experiments when the PPC concentration in the bottom of the gradient was high, a small CsCl pellet formed; it was discarded.

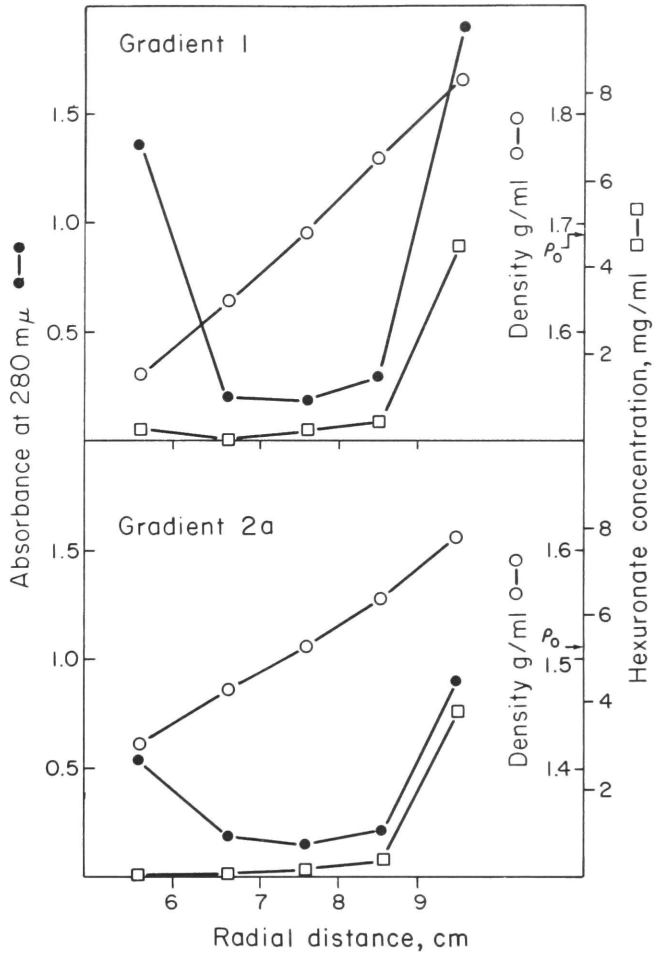

Figure 5. Analyses of density gradients used for preparation of proteinpolysaccharide complex, Gradient 1, and for preparation of proteoglycan subunit and glycoproten link, Gradient 2a. The ρ_0 indicates the initial density of each gradient.

link fraction free of glycoprotein-I fraction; the second provides a quick, one-step preparative method for obtaining PGS directly from the cartilage extract.

In the first method (density gradient 2a), PPC was dialyzed against 0.5 M guanidinium chloride buffered at pH 5.8. The retentate was then diluted with an equal volume of 7.5 M guanidinium chloride, which made the final solution 4 M in guanidinium chloride. CsCl was added to give a solution density of 1.50 g/ml (0.59 g CsCl per g solution). An equilibrium density gradient was formed by centrifugation at 40,000 rpm for 44 hours at 20°C in a Spinco SW 50.1 rotor. Fractions were isolated with a Spinco tube slicer. The densities, absorbance at 280 mμ and hexuronic acid content of each fraction were measured as described above. The results of such an analysis are shown in the bottom half of Figure 5. Most of the hexuronic acid and about 60% of the absorbance at 280 mμ contained in the PPC solution was recovered in the bottom two fractions of the gradient; the material in these fractions will be referred to as *proteoglycan subunit*, PGS. The material in the top fraction, which contained most of the remaining absorbance at 280 mμ, will be referred to as *glycoprotein link*, GPL. The second and third fractions from the top probably contain small amounts of material from both the GPL and PGS fractions; for analytical purposes they were discarded.

In the second method for preparing PGS (density gradient 2b), CsCl was added directly to a cartilage extract, which is already 4 M in guanidinium chloride, to give a density of 1.50 g/ml (0.59 g CsCl per g solution). An equilibrium density gradient was formed either in the SW 50.1 rotor by centrifugation at 40,000 rpm for 44 hours at 20°C or in a Spinco type 40 angle rotor by centrifugation at 34,000 rpm for 48 hours at 20°C. As in gradient 2a, PGS was isolated from the bottom 2/5 of the gradient. Unlike gradient 2a, however, the upper 1/5 of gradient 2b contained a surface gel and a *mixed glycoprotein* fraction with both GP-I and GPL present.

A cartilage extract contains 40-50% of the dry weight of the tissue. PGS comprises about 92% of the cartilage extract; the other components, GP-I, soluble collagen and GPL, each account for 2-3%. An outline of the extraction procedure and the different fractionation steps is shown in Table I.

5. Reaggregation experiments

a. Reaggregation of PGS and GPL -- A cartilage extract was dialyzed into 0.5 M guanidinium chloride buffered at pH 5.8; and a CsCl density gradient was established in the SW 50.1 rotor as in the preparation of PPC described above. The gradient was sliced into two fractions; the bottom 1/3 contained PPC and the top 2/3 contained GP-I.* The GP-I fraction was filtered through a glass wool plug to remove the small amount of collagen gel. It was then dialyzed against 4 M guanidinium chloride buffered at pH 5.8. Half of the PPC fraction was stored at 2°C, and the remainder was diluted with two volumes of 6 M guanidinium chloride to bring the solution to about 4 M guanidinium chloride. CsCl was added to give a density of 1.50 g/ml; and a density gradient identical to gradient 2a was established. The gradient was sliced into two fractions; the bottom 1/3 contained PGS and the upper 2/3 contained GPL. Two mixtures were made; a 2 ml aliquot of the GP-I solution was added to a 1 ml aliquot of PGS, and a 2 ml aliquot of the GPL solution was added to another 1 ml aliquot of PGS. These proportions approximated the ratio of each glycoprotein to PGS found in the cartilage extract. The mixtures were dialyzed twice against 10 volumes of 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8, along with separate aliquots of cartilage extract, PPC, PGS, GPL and GP-I. All samples except those which contained glycoprotein fractions only were adjusted

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* The gradients described in this experiment and those which follow were sliced 1/3 of the way from the bottom as a matter of convenience. This puts most of the small amounts of material in fractions 2 and 3 of the gradients depicted in Figure 5 above in the glycoprotein fractions. There would be little difference in the total amounts of material segregated into upper and lower fractions if the gradients were sliced anywhere between the upper 1/5 and the lower 2/5.

Table I

Density gradient methods for isolating fractions from bovine nasal cartilage extracts *

Extraction

1. 1 g tissue per 15 volumes 4 M guanidinium Cl, 24 hours, 20°C
2. filter

↓

Cartilage extract

1. dialysis, 0.5 M guanidinium Cl
2. plus CsCl, ρ_0 = 1.69 g/ml

↓

Density gradient 1

upper 1/5
- a. collagen gel
- b. GP-I

lower 2/5
- a. PPC

1. plus CsCl, directly, ρ_0 = 1.50 g/ml

↓

Density gradient 2b

upper 1/5
- a. collagen gel
- b. GP-I
- c. GPL

lower 2/5
- a. PGS

PPC

1. into 4 M guanidinium Cl
2. plus CsCl, ρ_0 = 1.50 g/ml

↓

Density gradient 2a

upper 1/5
- a. GPL

lower 2/5
- a. PGS

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* Abbreviations: GP-I, glycoprotein-I; PPC, proteinpolysaccharide complex; GPL, glycoprotein link; PGS, proteoglycan subunit

to the same hexuronic acid concentrations, equivalent to approximately 0.35% (w/v) in proteinpolysaccharide. The viscosities of the solutions were measured in a single-bulb Ubbelohde capillary viscometer because the 4-bulb viscometer described previously requires larger solution volumes than were available in this experiment. Thus, the data could not be corrected for shear and are tabulated as the apparent specific viscosity.

$$\eta_{sp}(app) = (t_s - t_0)/t_0 \qquad (7)$$

where t_s is the outflow time of the solution and t_0 is the outflow time of the solvent.

b. Reaggregation of PGS in the presence of different amounts of mixed glycoprotein -- A cartilage extract was prepared as described above with the exception that the cartilage slices were extracted with 10 volumes of 4 M guanidinium chloride solvent per gram of tissue rather than the 15 volumes ordinarily used. A density gradient identical to gradient 2b described above was established in the swinging bucket SW 50.1 rotor. The equilibrium gradient was sliced into two fractions; the bottom 1/3 contained PGS and the upper 2/3 contained a mixture of GP-I and GPL as well as a surface collagen gel. The gel was removed by filtration through a glass wool plug and was discarded. Both fractions were dialyzed against 10 volumes of 4 M guanidinium chloride, 0.05 M MES, pH 5.8 to remove most of the CsCl. They were then diluted with the same solvent to a final volume of 17 ml for the PGS fraction and 35 ml for the mixed glycoprotein fraction. Portions of the mixed glycoprotein from 0-7 ml were added to individual 2 ml aliquots of the PGS fraction, followed by sufficient 4 M guanidinium chloride solvent to bring the total volume of each mixture to 9 ml. The solutions were then dialyzed, first against 7 volumes of 0.05 M MES, pH 5.8 to bring the guanidinium chloride concentration in each to 0.5 M, and then against 10 volumes of 0.5 M guanidinium chloride in the same buffer. The hexuronic acid contents of the retentates were measured. Then, the retentates were all adjusted to have the same proteinpolysaccharide concentration, 0.35%

(w/v), by the addition of dialysate. The viscosities of the solutions were measured in the 4-bulb Ubbelohde viscometer, and the specific viscosities at zero rate of shear were calculated. Aliquots from several of the retentates were diluted to a concentration of 0.12% (w/v) and observed in the ultracentrifuge; g(s) profiles were calculated.

C. Results and Discussion

1. Dissociative extraction procedure

Figure 6 shows the rate of extraction of proteinpolysaccharide from nasal cartilage slices in two solvents, 4 M guanidinium chloride and 0.15 M KCl. The data are presented as the percentage of the total tissue hexuronic acid extracted as a function of time. The tissue slices were stirred at room temperature in the extracting media, and they did not change their shape or appearance during the experiment. Almost none of the tissue collagen is solubilized. However, with time, proteinpolysaccharide is solubilized from the slices. In 4 M guanidinium chloride, there is a rapid release of hexuronic acid into the solvent; and at later times a limit of about 85% of the total in the tissue is recovered in the medium. The proteinpolysaccharide that this amount of hexuronic acid represents constitutes about 45% of the dry weight of the tissue. In the same amount of time, the KCl solvent extracts a limit of only 20% of the tissue proteinpolysaccharide. The 4 M guanidinium chloride extracts the additional 65% because this solvent dissociates proteinpolysaccharide aggregates which exist in the tissue matrix whereas the 0.15 M KCl medium does not; dissociation allows the bulk of the macromolecules to escape from the tissue. Eighty-five per cent of the tissue proteinpolysaccharide can be extracted in the KCl solvent only if the tissue slices are exhaustively homogenized and the tissue matrix mechanically disrupted. In general, solvents which do not dissociate aggregate give the same extraction curve as the KCl solvent. The evidence for the existence of proteinpolysaccharide aggregates *in vivo* and a discussion of the parameters important for dissociative extraction methods are presented by Sajdera, 1969, and Sajdera and Hascall, 1969.

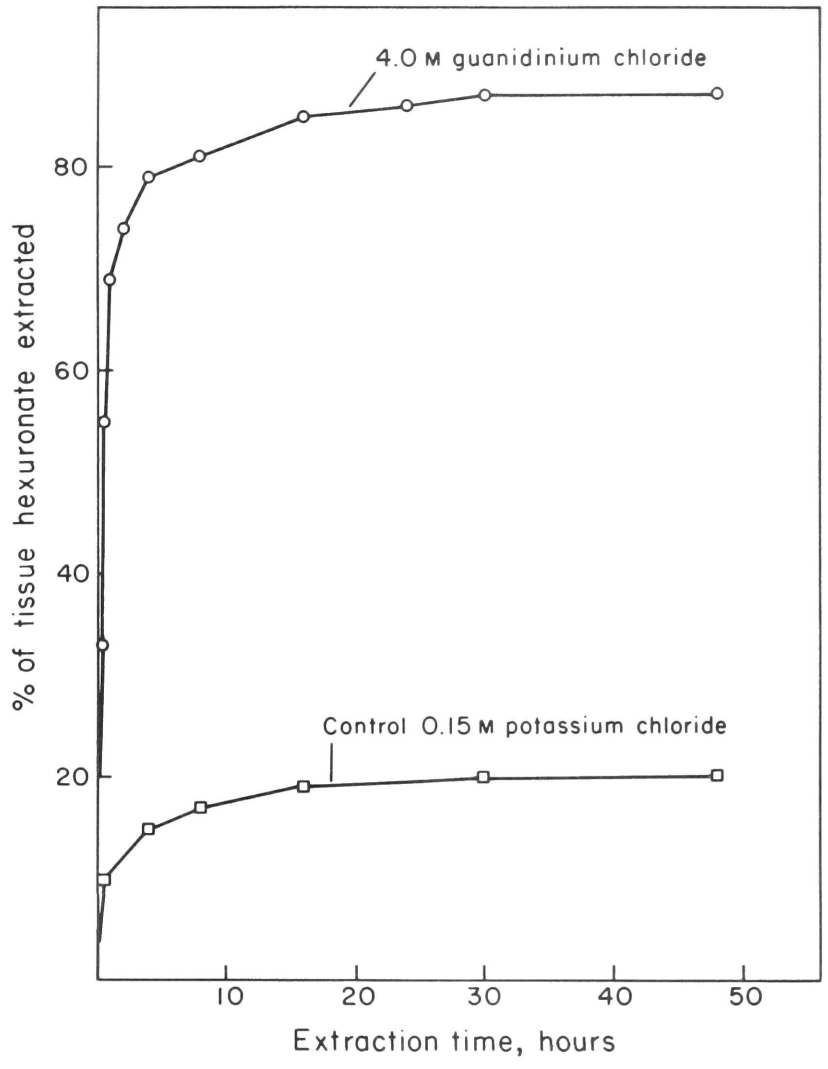

Figure 6. Kinetics of the extraction of proteinpolysaccharide from bovine nasal cartilage in two solvents, 4 M guanidinium chloride and 0.15 M KCl. The solutions (1 g tissue slices per 15 volumes of solvent) were stirred at 25°C. The amounts of hexuronic acid released into the media were measured at different times.

The 10-15% of the proteinpolysaccharide that is left behind in the slices after they have been extracted with 4 M guanidinium chloride appears to have the same chemical composition as that which was solubilized. All of the chondroitin sulfate chains in this insoluble fraction are extracted from the tissue if the slices are treated with 0.5 M NaOH for a few hours; this indicates that they are probably attached to proteins through glycosidic linkages to serines in the same way that chondroitin sulfate chains are attached to protein in the soluble proteinpolysaccharide fraction (see Figure 4 above). Therefore, these residual, insoluble macromolecules may be derived from the same basic proteinpolysaccharide subunit that constitutes the major portion of the soluble fraction; but they are more firmly, perhaps covalently, bound within the cartilage matrix. On the other hand, histological evidence from stained sections of extracted cartilage indicates that this residual pool is localized primarily in the territorial* matrix around the lacunae of the chondrocytes; the interterritorial matrix is largely devoid of proteinpolysaccharide (Sajdera, 1969). This makes it likely that the insoluble and soluble fractions in the cartilage are functionally distinct macromolecular pools even though both may have very similar compositions.

Figure 7 presents g(s) profiles obtained when a cartilage extract was analyzed in the ultracentrifuge. The upper curve shows the centrifugal behavior of the macromolecules in the extracting solvent, 4 M guanidinium chloride, and the lower curve shows the same solution after it was dialyzed against 0.5 M guanidinium chloride. Solute concentrations were about 0.15% (w/v). In the extracting solvent, there is one major, faster sedimenting peak and a small amount of a slower sedimenting material. After dialysis, these two components are still present. However, a third, more rapidly sedimenting peak has now appeared. Approximately 60% of the weight of the sample is sedimenting in this new peak.

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* See footnote on page 3.

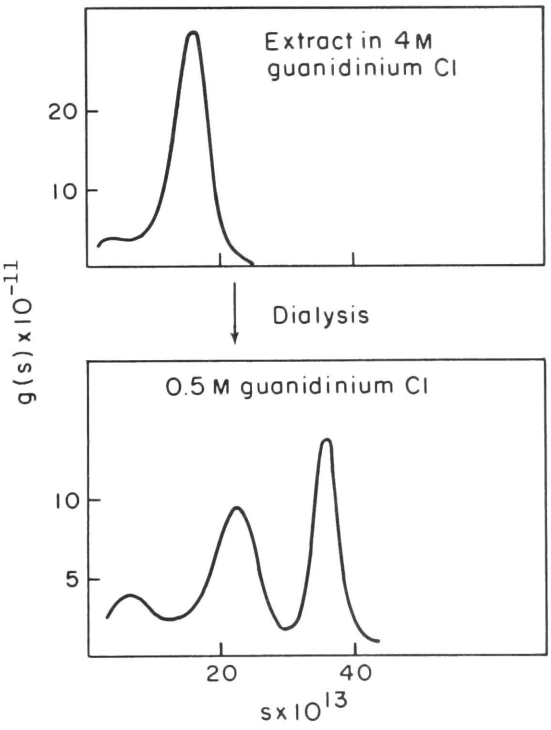

Figure 7. Sedimentation coefficient distributions, g(s), for a cartilage extract, first in the extraction medium, 4 M guanidinium chloride, and then after dialysis against 0.5 M guanidinium chloride. The solute concentrations were 0.15% (w/v). The solutions were buffered at pH 5.8 with 0.05 M MES.

Clearly, it must be an aggregate derived from macromolecules which sediment as the major component observed in the 4 M guanidinium chloride solvent. The 0.5 M guanidinium chloride is an *associative* solvent, *i.e.* one in which the macromolecules aggregate, while 4 M guanidinium chloride is a *dissociative* solvent. The fact that aggregation is reversible in these conditions is good evidence that non-covalent interactions between the macromolecules are responsible for aggregation because these solvent conditions would not be expected to make and break any covalent bonds.

2. Preparation of proteinpolysaccharide complex

The slowest sedimenting peak in the centrifugal patterns shown in Figure 7 for the dialyzed cartilage extract is a fraction which will be referred to as glycoprotein-I. It can be separated from proteinpolysaccharide by equilibrium centrifugation in a CsCl density gradient. CsCl was added to a dialyzed cartilage extract, and a density gradient was established in a swinging bucket rotor as is indicated in Figure 8 on the left. The sulfated polysaccharides have high densities; therefore, proteinpolysaccharide sediments to the bottom of the gradient. Low density substances float to the top, and the procedure provides a convenient way to purify proteinpolysaccharides. Ninety-five percent of the hexuronic acid is recovered in the bottom 2/5 of the gradient, which indicates that all but a few percent of the proteinpolysaccharide is recovered in this fraction. This preparation, referred to as proteinpolysaccharide complex, still contains aggregates. This is indicated by the centrifugal profile in the lower right of Figure 8. PPC was centrifuged in 0.5 M guanidinium chloride at a solute concentration of 0.20% (w/v). A profile for the dialyzed cartilage extract in identical conditions is shown at the upper right of the figure for comparison. The aggregate content has not been altered by the density gradient procedure; about 60% of the macromolecules still sediment in the faster peak. Additionally, the slowest peak in the dialyzed cartilage extract is no longer present in PPC. It was recovered in the upper 1/5 of the gradient as glycoprotein-I. GP-I contains about 30% of the soluble protein in the

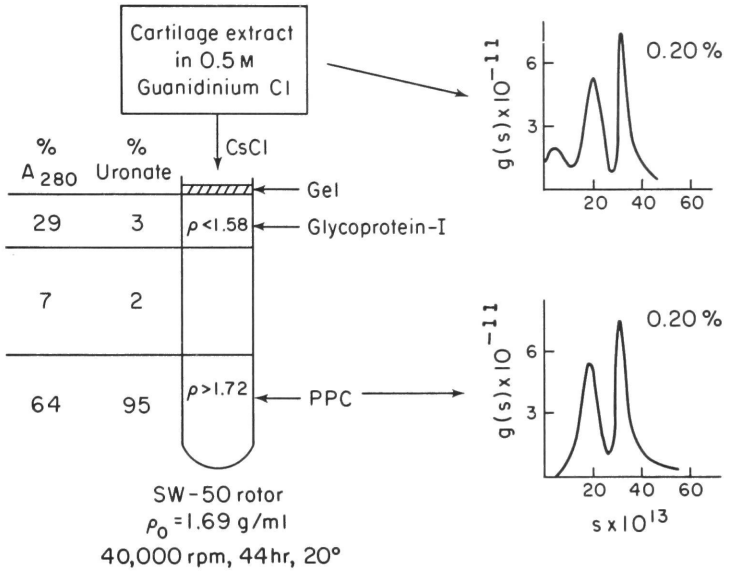

Figure 8. Equilibrium density gradient for preparing protein-polysaccharide complex, PPC. The gradient described on the left separated a dialyzed cartilage extract into three components: a collagen gel and glycoprotein-I were recovered at densities less than 1.58 g/ml and PPC was recovered at densities greater than 1.72 g/ml. The table at the left shows the percentage of absorbance at 280 mμ and the percentage of hexuronic acid recovered in the indicated fractions. The g(s) profiles at the right show the centrifugal properties of the cartilage extract and of PPC in 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8. The solute concentrations were 0.20% (w/v).

extract as is indicated by the percent of the absorbance at 280 mμ recovered in the upper fraction. This was subsequently verified when the total amino acid contents of GP-I and PPC were compared. A small amount of collagen formed a gel on the surface of the gradient.

3. Separation of proteoglycan subunit from glycoprotein link

PPC contains only about 10% protein and the remainder is polysaccharide. The results of the gradient described in Figure 8 indicate that PPC apparently consists of macromolecules with densities greater than 1.72 g/ml. However, this is not the case. PPC can be further fractionated in a second CsCl gradient formed at lower densities, but in the presence of 4 M guanidinium chloride, a solvent that dissociates the PPC aggregates. CsCl was added to a PPC preparation in 4 M guanidinium chloride, and a density gradient was established in a swinging bucket rotor as is indicated in Figure 9 on the left. At equilibrium, this gradient gave the analytical results shown. About 30% of the UV-absorbance, with only a small amount of hexuronic acid, is recovered in the upper 1/5 of the gradient in a fraction referred to as glycoprotein link, GPL. While this fraction contains about 1/3 of the total protein in PPC, it represents less than 3% of its total weight because PPC is predominantly polysaccharide. About 95% of the weight of the sample is recovered in the bottom 2/5 of the gradient in the proteoglycan subunit fraction. This is indicated by the high percentage of hexuronic acid recovered in the bottom. The high guanidinium chloride content of this second gradient has reversed a non-covalent interaction between the macromolecules in the GPL and PGS fractions. This PGS-GPL interaction was strong enough so that the lower density GPL molecules co-sedimented with the PGS molecules in the gradient in which PPC was prepared. This interaction appears to be essential to the aggregation process as is indicated by the ultracentrifugal studies shown in the right half of Figure 9.

Both the GPL and PGS fractions were dialyzed against 0.5 M guanidinium chloride buffered at pH 5.8 to remove excess CsCl and to bring

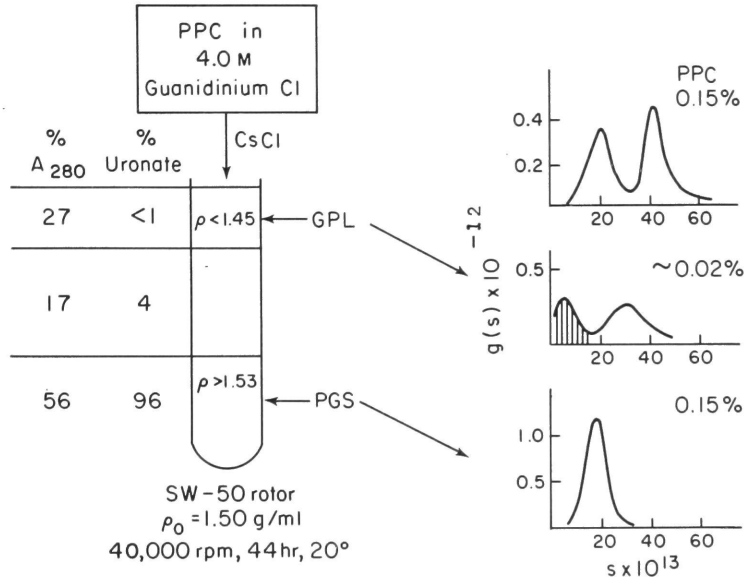

Figure 9. Equilibrium density gradient for preparing glycoprotein link, GPL, and proteoglycan subunit, PGS, from PPC. The gradient described on the left separated PPC into two components: GPL was recovered at densities less than 1.45 g/ml and PGS was recovered at densities greater than 1.53 g/ml. The table at the left shows the percentage of absorbance at 280 mμ and the percentage of hexuronic acid recovered in the indicated fractions. The g(s) profiles at the right show the centrifugal properties of PPC, GPL and PGS in 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8. The solute concentrations were 0.15% (w/v) for PPC and PGS, and approximately 0.02% (w/v) for GPL.

the solvent to ionic conditions which normally permit aggregation. They were then investigated in the ultracentrifuge as was an aliquot of PPC in the same conditions for comparison. The PGS and PPC solutions were diluted to 0.15% (w/v) whereas the GPL solution was centrifuged directly; the concentration of the GPL solution was estimated to be 0.02% (w/v) from the total fringe displacement of the interference patterns. The g(s) profiles were calculated and are presented in the right half of Figure 9. No aggregate is recovered in the PGS fraction, even though most of the weight of PPC is. This means that macromolecules which sedimented in the aggregate peak of PPC have either lost their ability to aggregate or a component required for aggregation has been removed. The fact that a small amount of a glycoprotein component, GPL, has been removed by the gradient procedure suggests that the latter interpretation is correct. The GPL fraction has a slow sedimenting peak (shaded); since no similar component is present in PPC, the molecules in this peak must have been tightly associated with the larger PGS molecules in the PPC preparation. It is likely that the molecules in this slow peak of the GPL preparation are specifically required before PGS macromolecules will aggregate.

There is a second, faster sedimenting component in GPL. Two observations suggest that this second peak contains PGS molecules which had not sedimented into the density gradient. First, the sedimentation coefficient of the peak is 30 Svedbergs at this low solute concentration, and this value is near the mean sedimentation coefficient of PGS at infinite dilution, 26 Svedbergs, as is shown in chapter IV below. Secondly, the small, but significant amounts of hexuronic acid and UV-absorbance recovered in the middle portion of the gradient indicate that some PGS molecules are floating into the gradient. This probably results from the fact that a guanidinium salt of PGS would be less dense than a cesium salt of PGS; and, in the high guanidinium concentration of the gradient, the competition of this counterion for the polyanionic sites within the PGS macromolecules decreases their buoyant densities. This

effect will be described further in chapter IV when it is used to partially fractionate the PGS preparation.

4. Reaggregation experiments

If the GPL fraction is required for aggregation, it should regenerate aggregate when it is recombined with PGS. Also, the amount of aggregate regenerated from a given aliquot of PGS should depend on how much GPL is present. The following experiments indicate that both of these conditions are satisfied.

a. Reaggregation of PGS and GPL -- The apparent specific viscosities of different fractions of a cartilage extract and of mixtures of PGS with either GPL or GP-I are listed in Table II. The solutions were prepared as described in Experimental Procedure. The apparent specific viscosity of the PPC solution was more than 2.5 times that of the PGS solution. Results described in the next section indicate that protein-polysaccharide solutions that have a greater amount of aggregate relative to subunit have greater viscosities when they have the same solute concentration and are in the same solvent. Thus, the higher viscosity of PPC with respect to PGS results from the presence of aggregate in the former solution. The dialyzed cartilage extract from which the PPC was prepared had essentially the same viscosity as that of the PPC solution. This indicates that the small amount of soluble collagen in the cartilage extract did not contribute significantly to the solution viscosity. The apparent specific viscosities of the two undiluted glycoprotein solutions, GPL and GP-I, were much less than that of the PGS solution. When the PGS and GPL solutions were combined in a ratio equivalent to their ratio in the PPC solution and then reaggregated, the apparent specific viscosity of the resulting mixture was more than twice that of PGS alone, or about 70% of the difference between PPC and PGS. This suggests that reaggregation in this case was about 70% efficient. When an aliquot of GP-I was substituted for GPL and reaggregated with PGS, quite different results were observed. The apparent specific viscosity of the mixture was only 10% higher than PGS alone, and less than 6% of the difference between

Table II

Apparent specific viscosities of fractions of cartilage extracts and mixtures of proteoglycan subunit with glycoprotein link and glycoprotein-I

Solutions[a]	$\eta_{sp}(app)$[b]
cartilage extract	2.12
PPC	2.21
PGS	.84
PGS + 2 volumes GPL	1.77
PGS + 2 volumes GP-I	.92
GPL	.04
GP-I	.09

[a] The hexuronic acid concentrations were: less than 0.03 mg/ml for GPL, 0.1 mg/ml for GP-I, and 1.0 mg/ml for the others. The solvent was 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8.

[b] $\eta_{sp}(app)$ is defined in equation 7 of Experimental Procedure.

PPC and PGS. This indicates that molecules in the GP-I fraction are ineffective in initiating aggregation with PGS.

b. Reaggregation of PGS in the presence of different amounts of mixed glycoprotein -- The results of the previous experiment indicate that the molecules which allow PGS to aggregate reside in the GPL fraction. They also indicate that those in the GP-I fraction are not involved in the aggregation process of PGS *in vitro*. For this reason, a mixed glycoprotein fraction, which contains both GP-I and GPL and which can be prepared directly from a cartilage extract, can be used in reaggregation experiments. PGS and mixed glycoprotein fractions were prepared as described in Experimental Procedure. Equal aliquots of PGS were added to solutions with different amounts of glycoprotein and the mixtures were dialyzed into 0.5 M guanidinium chloride to allow reaggregation. The retentates were diluted with dialysate to bring them all to the same solute concentration, about 0.35% (w/v) as estimated from their hexuronic acid contents. The large graph in Figure 10a indicates the specific viscosities at zero shear of the solutions as a function of the relative amount of glycoprotein to PGS in each. Figure 10b shows the g(s) distribution determined from centrifugal analyses of the solutions identified by Roman numerals I-IV on the left after they were diluted to a solute concentration of 0.12% (w/v).

Consider the centrifugal results first. The solution with the Roman numeral IV had the least amount of aggregate, designated by A, and the largest amount of subunit, designated by S. As the ratio of glycoprotein to PGS increased from bottom to top, the amount of aggregate increased at the expense of subunit. The centrifugal patterns indicate that the aggregate has a pronounced concentration dependence on its sedimentation velocities; the sedimentation coefficients of the aggregate increase with dilution. This point is discussed in more detail in chapter IV. The slowest sedimenting peaks in the patterns, which are partially obscured by the menisci, are glycoprotein-I (indicated by the G's). The size of this peak increased as the ratio of the mixed

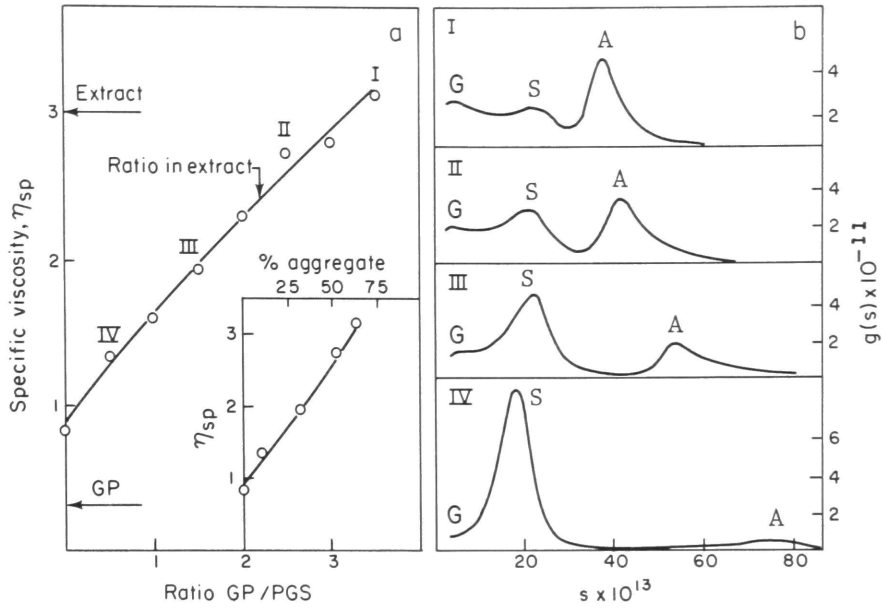

Figure 10. Reaggregation of PGS in the presence of different amounts of mixed glycoprotein, GP.

a. Solutions were prepared by adding different amounts of mixed glycoprotein to PGS and were then reaggregated. The large graph plots the specific viscosities of the mixtures against the ratio of glycoprotein to PGS in each. η_{sp} is defined in equation 6 of Experimental Procedure. The solute concentrations were 0.35% (w/v); the solvent was 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8. The arrows to the ordinate are the specific viscosities of an unfractionated extract under the same conditions (upper) and of the undiluted mixed glycoprotein solution (lower). The insert shows the specific viscosities of those solutions indicated by the Roman numerals plotted against the relative amount of aggregate to subunit in each (determined from the g(s) distributions in b).

b. The solutions indicated by the Roman numerals in part a were diluted to 0.12% (w/v) and analyzed in the ultracentrifuge; the g(s) profiles are presented for each. The subunit peaks are indicated by the S's, the aggregate peaks by the A's; the slowest sedimenting component, indicated by the G's, is glycoprotein-I.

glycoprotein to PGS increased, which is additional indirect evidence that GP-I apparently does not interact with PGS to form aggregate.

The increases in aggregate content with the concomitant decreases in subunit observed in Figure 10b correlate well with the increases in specific viscosities for the four solutions. This is illustrated by the small insert in Figure 10a. The percentage aggregate for the solutions was determined by comparing the areas under the subunit and aggregate peaks in the centrifugal patterns. The zero aggregate datum is the specific viscosity of a PGS solution under identical solvent conditions. These results indicate that the viscosities of proteinpolysaccharide solutions under defined conditions can be used as an estimate of aggregate content, and the large curve in Figure 10a is an alternative way of showing that the amount of aggregate in the solutions increased as the ratio of glycoprotein to PGS increased. The viscosities of the mixtures were greater than that of either the PGS solution, or of the undiluted glycoprotein solution, which is indicated by the lower arrow to the ordinate; they were, in fact, proportional to the ratio of glycoprotein to PGS (or GPL/PGS) which each mixture contained. The upper arrow to the ordinate is the specific viscosity of an unfractionated extract; the arrow on the curve indicates the ratio of glycoprotein to PGS that is found in an unfractionated extract. The closeness of the two values shows that reaggregation of the mixtures was fairly efficient. The data also suggest that all the macromolecules in the subunit peak are capable of aggregating because the increase in viscosity of the mixtures is linear with percentage of aggregate in the solution up to at least 65% aggregate content. It is possible, then, that the relative amount of aggregate to subunit in the tissue is mediated by the amount of glycoprotein link molecules available. However, the experiment does not rule out the possibility that the molecules in the slower sedimenting peak of PPC are incapable of aggregating even though they have the same sedimentation characteristics as those which can aggregate.

c. Reaggregation of PGS and mixed glycoprotein in an associative solvent -- In the previous two experiments the mixtures were prepared

in 4 M guanidinium chloride, a dissociative solvent, before reaggregation. The following experiment shows that aggregation can take place directly in associative solvents. Mixed glycoprotein and PGS were prepared as in the previous experiment. They were dialyzed separately against 0.5 M guanidinium chloride buffered at pH 5.8. Different amounts of the glycoprotein were added to equal aliquots of the PGS preparation and all the mixtures were adjusted with the 0.5 M guanidinium chloride solvent to the same volume. The final glycoprotein content of the solutions, relative to PGS, were between 0.4 and 1.2 times the ratio found in unfractionated extracts. The viscosities of these solutions and of appropriate controls are given in Table III. The viscosities of the mixtures increased with time and stabilized within 16 hours at the values given; again the final specific viscosities were proportional to the ratio of glycoprotein to PGS that each mixture contained. Aggregation under these conditions was efficient; the viscosity of the solution with a glycoprotein to PGS ratio of 1.2 was slightly higher than that of the unfractionated extract. The results show that the macromolecules retain their ability to interact and aggregate at lower ionic strengths. This could indicate that PGS macromolecules are in an equilibrium between subunit and aggregate in the matrix of the cartilage. The results also suggest that the first interactions *in vivo* between GPL and PGS may occur after the molecules have been synthesized, perhaps after they have been transported out of the chondrocytes into the matrix.

5. Chemical composition of the soluble fractions of a cartilage extract

The results from chemical analyses of the soluble fractions of a cartilage extract are given in Tables IV and V. The two glycoprotein fractions have amino acid profiles which are very similar to each other and to the glycoprotein isolated from bovine nasal proteinpolysaccharide by Franek and Dunstone, 1967, and Dunstone and Franek, 1967. The GP-I fraction still has an appreciable amount of collagen in it as is indicated by its hydroxyproline content. The high proportions of amino acids in the glycoprotein fractions indicate that they are primarily

Table III

Specific viscosities[a] of solutions made by combining mixed glycoprotein with proteoglycan subunit in an associative solvent.

	final hexuronate content (mg/ml)	ratio[b] of mixed glycoprotein to PGS	specific viscosity
cartilage extract	1.50	1.0	7.1
mixed glycoprotein only	0.22	-	0.4
proteoglycan subunit only	1.50	0.0	1.7
mixed glycoprotein plus PGS	1.54	0.4	3.2[c]
mixed glycoprotein plus PGS	1.59	0.8	5.9[c]
mixed glycoprotein plus PGS	1.64	1.2	7.7[c]

[a] Measurements were made in a four-bulb Ubbelohde viscometer at 25°C and specific viscosities were calculated from extrapolation to zero shear as described in Experimental Procedure; the solvent was 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8.

[b] A ratio of 1.0 is defined as the ratio of mixed glycoprotein to proteoglycan subunit found in an unfractionated extract.

[c] Values indicated were measured 16 hours after mixing, by which time further increases in the viscosities were negligible.

Table IV

Amino acid composition of fractions

residues per 1000

	GP-I	PPC	GPL	PGS
Hydroxyproline	10	--	--	--
Aspartic acid	119	80	115	68
Threonine	43	63	50	63
Serine	59	99	59	113
Glutamic acid	103	128	93	143
Proline	76	103	73	100
Glycine	113	93	76	101
Alanine	77	81	84	80
half-Cystine	20	11	15	9
Valine	43	67	67	65
Methionine	13	5	6	3
Isoleucine	33	33	32	34
Leucine	90	71	84	71
Tryosine	26	25	49	21
Phenylalanine	31	40	52	41
Lysine	66	30	50	23
Histidine	21	23	26	24
Arginine	55	47	69	38

Table V

Analytical composition of fractions

	Moles per 1000 moles[a]			
	GP-I	PPC	GPL	PGS
Hexuronic acid	92	389	39	408
Galactosamine	86	389	39	424
Glucosamine	29	42	61	35
Hexose	35	b	69	b
Sialic acid	2-3	4-5	5-6	4-5
Amino Acids	755	175	786	127
Ratio of galactosamine to glucosamine	2.9	9.3	0.63	12.1
Protein (% dry weight)[c]	-	9.5	-	6.6

[a] Values are based on the sum of the moles of the components presented in the table normalized to 1000.

[b] Values are not given; background from other carbohydrate components was too high for reliable estimation of hexose.

[c] Values are based on the total amount of amino acids per hexuronic acid content and the measured value of 25.7 mg glucuronolactone per 100 mg of dry weight of guanidinium-PGS.

protein. They also have higher ratios of glucosamine to galactosamine than the PPC or PGS fractions, which suggests that they contain relatively more keratan sulfate to chondroitin sulfate. The PPC and PGS are predominantly polysaccharide, most of which is chondroitin sulfate; and they have only about 10% and 7% protein content respectively. When PPC is fractionated into PGS and GPL, significant differences are observed. Serine, glutamic acid, proline and glycine are proportionately higher in PGS than in PPC. Aspartic acid, the basic amino acids and glucosamine are higher in the GPL fraction. The four amino acids which are relatively more abundant in PGS are those which are thought to reside in the attachment region between the polypeptide and polysaccharide components of the proteinpolysaccharides (Anderson, Hoffman and Meyer, 1965). Although PGS has less glucosamine than PPC, it still has enough to suggest that about 8% of the polysaccharide in the subunit is keratan sulfate.

The results described in this section offer an explanation for the variable hexosamine ratios and protein contents observed in proteinpolysaccharide preparations such as those reported by Pal, Doganges and Schubert, 1966. Their procedure describes centrifugal methods for partitioning the soluble proteinpolysaccharide pool from bovine cartilage into 4 separate fractions, which they call PP-L3 through PP-L6. Their data indicate that the preparations with higher protein contents also have 1) higher glucosamine to galactosamine ratios, 2) relatively more aspartic acid and basic amino acids, and 3) fewer of the attachment region amino acids serine, glutamic acid, proline and glycine. These data indicate that the different fractions have different amounts of glycoprotein associated with them.

The analytical data given for PGS in Table V can be used to predict the weight percentage of a PGS preparation which would be glucuronic acid. The data suggest that an hypothetical guanidinium-PGS molecule with 408 repeating disaccharides of chondroitin sulfate (the value for hexuronic acid content) would have about 35 repeating

disaccharides of keratan sulfate (the value for glucosamine content) and 127 amino acid residues. The average molecular weight of a residue in a polypeptide with the composition given for PGS in Table IV is 106. In addition, there would be about 10 attachment region oligosaccharides between protein and chondroitin sulfate of the type drawn in Figure 3 above. These values and the values of the molecular weights of the different constituents indicate that glucuronic acid (calculated as glucuronolactone) should constitute 26.4% of the preparation. Similarly, glucuronic acid would constitute 25.4% of a guanidinium salt of PPC and 30.4% of a guanidinium salt of pure chondroitin sulfate.

The amount of glucuronic acid per dry weight of PGS was measured in a separate experiment. A PGS preparation was dialyzed 3 times against 10 volumes of 1 M guanidinium chloride and then exhaustively against water to remove all excess guanidinium chloride. The preparation was lyophilized and then dried over phosphorous pentoxide *in vacuo* at 78°C for 15 hours. The amount of hexuronic acid (as glucuronolactone) in the preparation was found to be 25.7% of the dry weight, in close agreement with the calculated value. This result justifies, to a large extent, the use of hexuronic acid content as an estimation of the proteinpolysaccharide concentration in a sample. Thus, the value of 100 mg dry weight per 25.7 mg hexuronic acid (measured as glucuronolactone) was used to calculate proteinpolysaccharide concentrations in the different solutions described.

6. General discussion and conclusions

The aggregates contained in a proteinpolysaccharide complex preparation are composed of a number of proteoglycan subunit macromolecules bound together via non-covalent interactions with molecules in the glycoprotein link fraction. Since the analytical composition of GPL indicates that it is predominantly protein, it is postulated that the effective agent(s) for mediating aggregation is a protein or glycoprotein. The structural significance of the GPL fraction, which comprises less than 3% of PPC, is demonstrated by the facts that aggregation of

PGS does not occur in its absence and that the amount of aggregate formed from an aliquot of PGS depends upon the amount of GPL in the solution. The proteoglycan subunit contains about 80% of the ground substance material of the tissue, or almost 45% of the dry weight of the cartilage. It appears to be a single class of macromolecules, although, as will be seen in chapter IV, it is very polydisperse and has a large range of molecular weights. The gradient methods used to prepare PGS make it likely that all the chemical constituents of the macromolecules are covalently attached. This means that the chemical structure of PGS is complicated, with protein, chondroitin sulfate and keratan sulfate all synthesized into one large molecule. The convenient and rapid preparation of PGS and the fact that it represents a major fraction of the proteinpolysaccharides of the tissue make it ideal for further structural and biosynthetic studies for this class of macromolecules.

The experiments described in this chapter used proteinpolysaccharide isolated from bovine nasal cartilage. It is probable, however, that the mechanisms involved in the aggregation of PPC will be similar for proteinpolysaccharides prepared from a variety of cartilaginous tissues. Sajdera, 1969, described extraction experiments with bovine costal cartilage and shark cartilages which indicate that these tissues may have proteinpolysaccharide aggregates in their extracellular matrices. Also, the nucleus pulposus, a spongy tissue in the intervertebral discs, which is physically quite dissimilar to nasal cartilage, apparently contains proteinpolysaccharide aggregates that require linking molecules. This is indicated, albeit indirectly, by results reported by two laboratories. Hashimoto and Ludowieg, 1968, investigated samples of proteinpolysaccharide from whale nucleus pulposus in the ultracentrifuge; their preparations contained two peaks. The faster sedimenting one was irreversibly disaggregated by treatment with dithiothreitol, an effective sulfhydryl reducing agent. The next chapter demonstrates that such treatment abolishes aggregation of PPC. Further, Rosenberg, Schubert and Sandson, 1967, fractionated bovine

nucleus pulposus proteinpolysaccharide by the methods of Pal *et. al.*, 1966. The composition of the nucleus pulposus fractions, PP-L3 through PP-L6, were strikingly similar to those of bovine nasal cartilage. The fractions showed the trends in amino acid composition which are consistent with the presence of a link glycoprotein; basic amino acids and aspartic acid increased as protein content of the fractions increased with a concomitant decrease in the four amino acids near the attachment region between protein and polysaccharide; serine, proline, glycine and glutamic acid.

Other workers have proposed that the non-collagenous matrix of cartilage contains heteroaggregates of large proteinpolysaccharide molecules and much smaller glycoprotein 'bridging' molecules. The first workers to make such a suggestion were Mathews and Lozaityte, 1958. They reported physical measurements of proteinpolysaccharides from nasal cartilage and pointed out that the macromolecules tended to aggregate. They also observed a variable protein content in their different preparations and suggested "the possibility that a small amount of extraneous protein may participate in the formation of aggregates." This suggestion was largely overlooked until Fitton-Jackson, 1964, and Fitton-Jackson, 1965, revived interest in glycoproteins and aggregation. She used electron microscopic techniques to investigate a proteinpolysaccharide preparation which was obtained from brief aqueous extracts of chick epiphyses. She inferred from her observations that the macromolecules were aggregates of about 6 proteinpolysaccharide subunits which were possibly organized through non-covalent associations around a protein or glycoprotein core. This preparation had a much higher protein content (60%) and lower sedimentation velocities (20 Svedbergs at zero concentration) than PGS. Thus, if this preparation is an aggregate, the subunits must be much smaller than PGS and the organization of the aggregate different from that in PPC. It is possible that application of the extraction and gradient techniques described in this chapter may provide direct evidence of glycoprotein-mediated aggregation of proteinpolysaccharides in chick epiphyses.

Franek and Dunstone, 1967, and Partridge, Whiting and Davis, 1965, have isolated glycoprotein fractions from bovine nasal proteinpolysaccharide with equilibrium density gradient centrifugation and anion exchange chromatography, respectively. Following the suggestion of Fitton-Jackson, these workers proposed that the isolated glycoprotein might serve as 'bridging' molecules between proteinpolysaccharides in the tissue. The density gradient used for the preparation of PPC that was presented in this chapter was patterned after that described by Franek and Dunstone; in addition, the proteinpolysaccharide they isolated in the bottom of their gradients contained two peaks when it was observed in the ultracentrifuge. These facts suggest that the glycoprotein fraction they isolated is similar to GP-I, which is not involved in the aggregates of PPC *in vitro*. The column fractionation method used by Partridge *et. al.* and described further by Partridge, 1968, is harder to evaluate. It is possible that the anion exchange resin dissociated proteinpolysaccharide aggregates and allowed them to separate GPL from PGS, but the authors present no evidence that their isolated glycoprotein fraction could initiate aggregation. Additionally, the proteinpolysaccharide preparation that they isolated from the column after glycoprotein had been removed, had physical characteristics which suggest that the macromolecules had been extensively depolymerized; the molecular weights of the products were only about 250,000 and the sedimentation coefficients were not much greater than isolated chondroitin sulfate chains. These physical properties are quite different from those of PGS which are described in chapter IV. Because the column techniques these workers used required long periods of time, they added diisopropylfluorophosphate, DFP, to their samples to prevent autolysis or bacterial contamination. However, proteinpolysaccharides have a large number of unsubstituted serines in their protein cores, and DFP may react with many of them. These newly phosphorylated serines would be reactive and might subsequently be involved in some specific or non-specific cleavage reaction which would result in the small molecular weight products recovered from the column.

In conclusion, the experiments discussed in this chapter suggest that cartilage contains glycoprotein which functions as a linking molecule in the formation of proteinpolysaccharide aggregates. A method for preparing proteoglycan subunit, which appears to be the fundamental structural component of the ground substance of the cartilage, is described. The hypothesis is suggested that the PGS macromolecules are organized within the tissue matrix through glycoprotein-mediated interactions either with other PGS macromolecules or with insoluble elements of the matrix. This hypothesis is consistent with the long standing observations that the proteinpolysaccharides are tightly associated with or within the insoluble fibrous collagen matrix.

Chapter III

PROPERTIES OF THE INTERACTIONS REQUIRED FOR AGGREGATION OF PROTEINPOLYSACCHARIDE

A. Introduction

Meyer, Palmer and Smyth, 1937, observed that chondroitin sulfate co-precipitated in acidic solutions with a number of proteins, including gelatin. Subsequently, Meyer and Smyth, 1937, reported that 10% $CaCl_2$ extracted a significant amount of the chondroitin sulfate in cartilage. They felt that the mechanism of extraction depended upon reversing ionic interactions between the polyanion, chondroitin sulfate, and basic groups on proteins in the matrix, possibly on collagen. They concluded that the "major portion of the cartilage is a protein salt of chondroitin sulfuric acid." Later, a number of laboratories, most notably that of Schubert, showed that the chondroitin sulfate in cartilage is primarily, if not exclusively, attached covalently to non-collagenous protein to form the large macromolecules which are currently called proteinpolysaccharides. Schubert, 1958, has reviewed the evidence from earlier work for the covalent structure of the proteinpolysaccharides; he also discussed results which indicated that the primary counterion for chondroitin sulfate is probably sodium. More recently, however, the suggestion that ionic interactions between chondroitin sulfate and protein are important for the structure of the ground substance has been revived in slightly modified forms. Partridge, 1968, postulated that proteinpolysaccharide subunits with molecular weights about 250,000 are bound into large, net-like aggregates in the tissue matrix through associations with small, globular proteins which act as bridges between portions of the chondroitin sulfate moieties on adjacent subunits. At the same time, Mathews, 1968b, presented evidence that soluble collagen can specifically associate with chondroitin sulfate, and he proposed a structure for cartilage in which some of the chondroitin sulfate in proteinpolysaccharide molecules is ionically associated with collagen.

It seemed possible, even probable, that the aggregation of GPL and PGS might result from similar ionic interactions between polysaccharide and protein, particularly since the effectiveness of extraction of the macromolecules from the cartilage depended upon the use of concentrated

salt solutions. A series of experiments were undertaken to test this possibility and to determine some of the characteristics of the interactions between GPL and PGS that are required for aggregation. The experimental approach employed was to test a number of reagents and solvents to see what conditions dissociate aggregates in PPC preparations; both viscometry and centrifugation were used as assay procedures.

Somewhat surprisingly, the data indicate that the interactions between GPL and PGS are mediated by protein conformations and probably involve direct protein to protein associations. The results also suggest that both GPL and PGS are at least bifunctional with two or more chemically distinct interaction sites within each.

B. Experimental Procedure

1. Materials

N-ethylmaleimide and iodoacetamide were purchased from K and K Laboratories. Dithiothreitol, A grade, was obtained from Calbiochem. Other reagents and the preparation of tissue slices were described in Experimental Procedure, chapter II.

2. Preparation of PPC and PGS

PPC was prepared from dialyzed cartilage extracts by the density gradient methods described in the previous chapter. A Spinco type 40 angle rotor was used to establish the gradients. PPC was isolated from the bottom 2/5 of the tubes and was dialyzed into the buffers used in the experiments outlined below.

PGS was prepared by adding CsCl directly to cartilage extracts and establishing a density gradient in the Spinco type 40 angle rotor (described as gradient 2b in the previous chapter). PGS was recovered from the bottom 2/5 of the gradient. In some experiments the tubes were sliced 1/3 of the distance from the bottom and the mixed glycoprotein fraction, GP-I plus GPL, was recovered from the upper 2/3. PGS and mixed glycoprotein were dialyzed into the buffers used in the experiments outlined below.

3. The effect of extraction pH on aggregation

The effect of the pH of the extracting medium on the aggregation process was determined as follows. Aliquots of 3 M guanidinium chloride* were made 0.05 M in either sodium formate, sodium acetate, sodium cacodylate, or Tris base. Aliquots of these solvents were titrated with concentrated HCl to pH 3.8 and 4.4 (sodium formate), pH 4.9, 5.4 and 5.8 (sodium acetate), pH 6.5, 7.0 and 7.5 (sodium cacodylate), and pH 8.0 and 8.4 (Tris base). Two gram samples of cartilage slices were then extracted with 30 ml portions of each of these solutions for 24 hours at room temperature. The extracts were freed of slices and particulate matter by vacuum filtration with the aid of 5% (w/v) Hyflo Super-Cel and the filter cakes were discarded without washing. A 20 ml aliquot of each of the extracts was dialyzed overnight against 100 ml of 0.05 M buffer of the same type and pH as that of the particular extract. This brought the retentates to 0.5 M in guanidinium chloride and allowed the extracts to reaggregate at the pH of the extraction step. All samples were then dialyzed against a large volume of 0.5 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8 in order to bring them all to identical solvent conditions. The retentates were adjusted with dialysate to the same solute concentration, 0.45% (w/v), and their specific viscosities at zero shear were determined as described in Experimental Procedure of the previous chapter.

4. The effect of solvent pH on the stability of aggregate

Samples of PPC and PGS from the bottom 2/5 of their respective density gradients were dialyzed against 0.5 M guanidinium chloride, 0.005 M MES, pH 5.8. The retentates were diluted with dialysate to have identical solute concentrations, 0.75% (w/v). A 5 ml aliquot of either the

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* 3 M guanidinium chloride is as effective a dissociative solvent as 4 M guanidinium chloride; both extract the same amount of proteinpolysaccharide from cartilage in 24 hours (Sajdera and Hascall, 1969).

PPC or PGS solution was added to a 5 ml aliquot of 0.5 M guanidinium chloride in one of the following buffers: 0.1 M formate, pH 2.3; 0.1 M acetate, pH 3.4; 0.1 M acetate, pH 4.4; 0.1 M MES, pH 5.8; or 0.1 M tris-HCl, pH 8.4. The resulting solution was then filtered into the Ubbelohde viscometer, and its specific viscosity determined. For those solutions which showed temporal decreases in viscosity, the specific viscosity was redetermined after 16 hours.

5. Effect of guanidinium chloride concentration on aggregation

Preparations of PPC and PGS were dialyzed into 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8. Both retentates were adjusted to the same solute concentration, 0.6% (w/v). A 5 ml aliquot of an appropriate guanidinium chloride solution (0.0 to 7.5 M) was added to an equal volume of either the PPC or the PGS solutions with concomitant mixing on a vortex stirrer. The resulting solution (0.25 to 4.0 M in guanidinium chloride) was then filtered into the Ubbelohde viscometer and its specific viscosity was determined. The measurements were repeated after 24 hours for those solutions which showed temporal decreases in viscosity.

6. Effect of reduction on aggregation

a. Reduction and alkylation of PPC in 0.4 M guanidinium chloride -- A sample of PPC was prepared as described above. It was diluted to a concentration of about 0.5% (w/v) and then was dialyzed against 0.4 M guanidinium chloride, 0.05 M tris-HCl, pH 8.5. An aliquot of the retentate was set aside for control experiments. The remainder was deoxygenated with nitrogen, and dithiothreitol was added to a final concentration of 10 mM. After incubation at 37°C for 16 hours, iodoacetamide was added to the solution to a final concentration of 40 mM, and alkylation was allowed to proceed for 4 hours in the dark at room temperature. An aliquot of PPC which had not been incubated with dithiothreitol was treated with iodoacetamide in the same way. This unreduced but alkylated sample of PPC and an aliquot of reduced and alkylated PPC were then dialyzed against 0.1 M KCl to remove guanidinium ion. The

retentates were hydrolyzed in the absence of oxygen for amino acid analysis. The S-carboxymethylcysteine and half-cystine contents of each were determined on the amino acid analyzer in order to estimate the extent of reduction (Crestfield, Moore and Stein, 1963). The remainder of the reduced and alkylated PPC was dialyzed against 0.4 M guanidinium chloride to remove excess alkylating reagent. The retentate was brought to a density of 1.55 g/ml by the addition of CsCl. A density gradient was formed in a SW 50.1 rotor by centrifugation at 40,000 rpm for 40 hours at 20°C. The gradient was sliced into five fractions, and the density of each was measured. The fractions were dialyzed against 0.4 M guanidinium chloride to remove cesium ion, and the absorbance at 280 mμ and the hexuronic acid content of each was measured. The fraction from the bottom of the gradient was then diluted with 4 volumes of 5 M guanidinium chloride to bring the final guanidinium chloride concentration in the solution to about 4 M. CsCl was added to give a solution density of 1.52 g/ml, and a second equilibrium density gradient was formed as above. This second gradient was analyzed for density, UV-absorbance and hexuronic acid as for the first gradient.

In a separate experiment an aliquot of the untreated PPC was diluted with 0.4 M guanidinium chloride, 0.05 M tris-HCl, pH 8.5 to a solute concentration of 0.18% (w/v). A centrifuge cell with a 30 mm Epon double-sectored centerpiece was loaded with 1.0 ml aliquots of the PPC solution and of solvent. The sample was centrifuged, and a g(s) profile was computed. Without disassembling the cell, 10 μl of 1.0 M dithiothreitol was added to the solution side. The cell was incubated at 37°C for 2 hours. The sample was then recentrifuged and a second g(s) profile was computed.

b. Reduction and alkylation of PPC in 4 M guanidinium chloride -- PPC was prepared as described above. It was diluted to a solute concentration of about 0.5% (w/v) and was dialyzed against 4 M guanidinium chloride, 0.05 M tris-HCl, pH 8.5. The solution was deoxygenated with nitrogen, and dithiothreitol was added to give a final concentration of 10 mM. The solution was incubated for 4 hours at 37°C. Iodoacetamide

was then added to give a final concentration of 40 mM, and alkylation was allowed to proceed for 4 hours at room temperature in the dark. Excess alkylating reagent was removed by dialyzing the solution against 10 volumes of 4 M guanidinium chloride. Part of the reduced and alkylated PPC was then dialyzed against 10 volumes of 0.05 M tris-HCl, pH 8.5, in order to bring the guanidinium chloride concentration to about 0.4 M. CsCl was added to the dialyzed sample and to a sample which was still 4 M in guanidinium chloride; the final densities of the two solutions were 1.55 g/ml. Density gradients were formed and analyzed.

c. Differential reduction of PGS and GPL -- A mixed glycoprotein fraction and a PGS fraction were prepared from a cartilage extract as described in section 2 above. The two fractions were dialyzed against 4 M guanidinium chloride, 0.05 M MES, pH 5.8, to remove excess CsCl. The pH value of 5.8 was used in this experiment to insure maximal recovery of aggregate (see section 1 of Results and Discussion). Aliquots of each of the two fractions were deoxygenated with nitrogen and incubated for 2 hours at 37°C, either with or without 10 mM dithiothreitol; control samples of unfractionated cartilage extract in the same solvent were treated similarly. Solid N-ethylmaleimide was added to all solutions to give a final concentration of 40 mM, and alkylation was allowed to proceed for one hour at room temperature. N-ethylmaleimide was used in this experiment because it is an effective alkylating reagent at pH 5.8 (Gregory, 1955) whereas iodoacetamide is not. The reduced and alkylated solutions and the alkylated controls were dialyzed against 4 M guanidinium chloride, 0.05 M MES, pH 5.8, to remove excess alkylating reagent. A series of test mixtures were prepared from the various retentates by adding one part of a PGS solution to two parts of a mixed glycoprotein solution in different combinations, such as reduced PGS with unreduced glycoprotein or unreduced PGS with reduced glycoprotein. This ratio of PGS to glycoprotein approximated that in the original extract. These mixed aliquots and appropriate controls were then dialyzed against 7 volumes of 0.05 M MES, pH 5.8, in order to bring the guanidinium chloride concentration to 0.5 M and to allow aggregation of

the samples. Each retentate was adjusted to the same concentration, 0.45% (w/v), with dialysate; and the specific viscosity of each was determined.

C. Results and discussion

1. Effect of extraction pH on aggregation

Cartilage extracts were made in 3 M guanidinium chloride solvents buffered at different pH values, and then were reaggregated at the pH value of the extraction step as is described in Experimental Procedure. The percentage of the total tissue proteinpolysaccharide extracted was the same for all the solvents, approximately 85%. Also the extracts contained equivalent, small amounts of soluble collagen as estimated from their hydroxyproline contents. The specific viscosities of the solutions are plotted in Figure 11 as a function of the pH of the extracting media. As was shown in Figure 10 in the last chapter, the viscosities of proteinpolysaccharide solutions depend upon the relative amount of aggregate in the sample; and a solution with a higher specific viscosity for a given amount of proteinpolysaccharide has more aggregate. Because all the solutions in this experiment had identical solute concentrations, the results indicate that extraction at pH 5.8 allowed a maximum amount of aggregate to be regenerated. Centrifugal analyses indicated that the solution which had been extracted at pH 5.8 contained about 60% aggregate while those which had been extracted at pH 3.8 and 8.4 contained only about 25-30% aggregate. All subsequent experiments started with cartilage extracts made at pH 5.8 in order to optimize recovery of aggregate.

2. Effect of solvent pH on the stability of aggregate

This experiment differs from the one just described in that the cartilage extract was made at pH 5.8 and reaggregated by dialysis against 0.5 M guanidinium chloride, 0.005 M MES, pH 5.8, before the solvent pH values were changed. Thus, this experiment was designed to test the stability of aggregate that had already been formed to different solvent pH values.

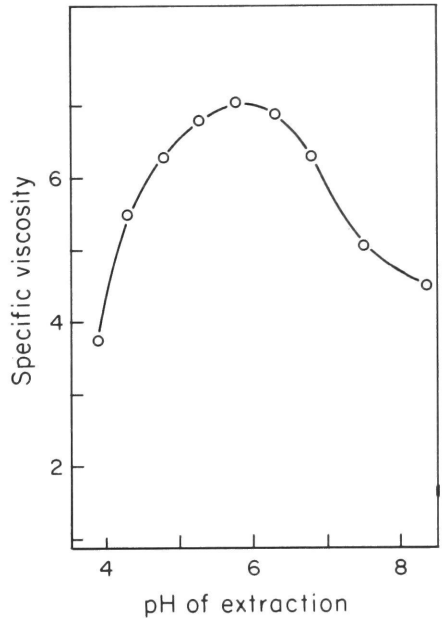


Figure 11. The effect of the pH of the extracting medium on recovery of aggregate. Cartilage extracts were prepared in 3 M guanidinium chloride buffered at the pH values indicated by the abscissa. They were reaggregated as described in Experimental Procedure, and their specific viscosities were measured. The solute concentrations were 0.45%; the solvent, in which the viscosities were measured, was 0.5 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8.

Figure 12 shows the specific viscosities of PPC (open circles) and of PGS (solid circles) in 0.5 M guanidinium chloride after the solvent pH was changed from pH 5.8 to those values indicated by the abscissa. The solid arrows indicate the decreases in the specific viscosities of the PPC solutions at pH 3.4 and 4.4 from the values first observed at these pHs (indicated by the bars) to the values observed 16 hours later. The results indicate that aggregate is stable to solvent pH values up to 8.4, but that it dissociates if the solvent pH is lowered. Dissociation is complete within 16 hours at pH 3.4 or below; the rate of dissociation was too rapid to detect at the lowest pH investigated, pH 2.3. The dashed arrow represents a control experiment to test if the dissociation of the aggregates in PPC by low pH is reversible. A PPC solution was titrated to pH 2.6 with formic acid in order to dissociate the macromolecules. After 30 minutes, the pH of the solution was brought to pH 6.0 with saturated Tris base. The specific viscosity of the solution was then determined; it increased over the range indicated during the following 16 hours. This result indicates that the dissociation which results from low solvent pH is, to a large extent, reversible. Centrifugal analyses corroborated the viscosity data. A PPC solution that was examined in the ultracentrifuge in the pH 2.6 solvent had one peak that sedimented with the same characteristics as a PGS solution in the same solvent; after titration to pH 6.0 the solution regenerated about 40% aggregate.

The transition between aggregation and disaggregation occurred over a fairly broad range of pH values, from pH 3 to 5. This probably indicates that carboxylate groups located in the protein or polysaccharide moieties of the macromolecules are necessary for the interactions which lead to aggregation and that the interactions are, in part, ionic. Keratan sulfate has no acidic groups which can be titrated in this pH range, nor does it have any basic groups. It is unlikely, then, that keratan sulfate could be directly involved in this interaction which is reversed by low solvent pH. Some evidence discussed below also makes it unlikely that chondroitin sulfate is directly involved either, although

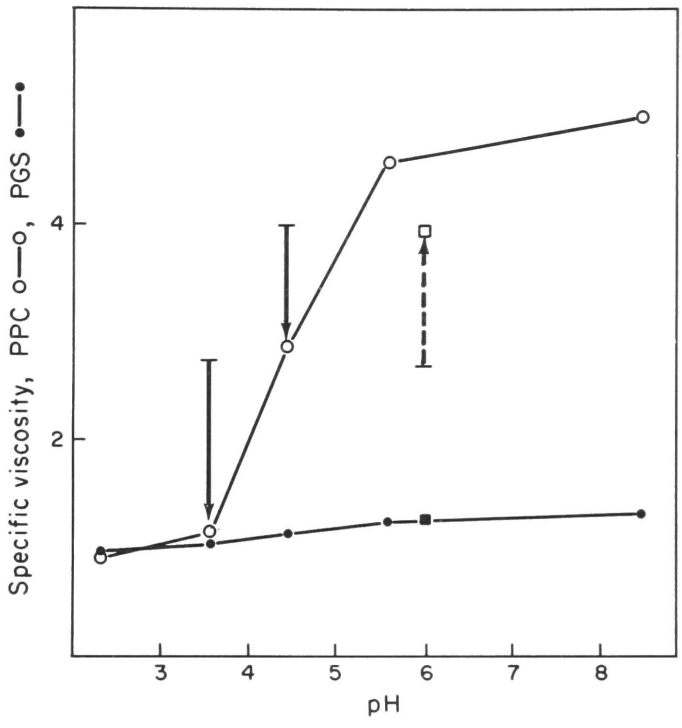


Figure 12. Disaggregation of PPC in solutions with low pH values. The graphs show the specific viscosities of PPC (open circles) and PGS (solid circles) in 0.5 M guanidinium chloride after the solvent pH was changed from pH 5.8 to the values indicated by the abscissa. The solid arrows show the decreases in viscosity for PPC at pH 3.4 and 4.4; the decreases occurred in the 16 hours after the solutions were prepared. The dashed arrow indicates the increase in viscosity observed during the 16 hours after a PPC solution was titrated to pH 6.0 after a 30 minute exposure to pH 2.6; the solid square was the specific viscosity for a PGS sample treated in the same manner. The solute concentrations were 0.38%.

this polysaccharide does have a carboxyl group in its repeating disaccharide structure. For these reasons, any carboxylate groups involved in the aggregation are probably located in the protein portions of the macromolecules. The proteins in PGS and GPL do have significant amounts of glutamic acid and aspartic acid residues, but it is not known how many of these residues have free carboxyl functions.

It is somewhat surprising that the viscosity of PGS does not show much dependence on solvent pH. At pH 5 or above, half of the negative charges in the PGS macromolecules are carboxylates in the chondroitin sulfate chains; at pH 2.3 most of these carboxylates are protonated. Contrary to what might be expected, titration of these carboxyl groups apparently does not greatly change the hydrodynamic characteristics of the macromolecules. Any large changes in the size or shape of the macromolecules would affect the viscosity, yet the viscosity of PGS is fairly insensitive to solvent pH and does not show any transition near pH 3.2, the pK of glucuronic acid (Haug and Larsen, 1961).

3. Effect of guanidinium chloride concentration on aggregation

Aliquots of PPC and PGS solutions with identical solute contents were diluted with equal volumes of different guanidinium chloride solvents and the viscosities of the resulting solutions were measured. The solid lines in Figure 13 show the specific viscosities of the PPC and PGS solutions as a function of the final electrolyte concentration; the ratio of the two values, PPC/PGS, is given by the dashed line. The amount of aggregate left in a PPC solution at a particular ionic strength is indicated by the difference between its viscosity and that of the PGS solution at the same ionic strength. Thus, a ratio of PPC/PGS equal to 1 indicates complete dissociation, and ratios with higher values indicate a larger percentage of aggregate in the PPC solutions. The proportion of aggregate in the PPC solution did not increase when the electrolyte concentration was lowered from 0.5 M to 0.25 M. This is indicated by the fact that the ratio of viscosities of PPC to PGS is no greater for the solutions in 0.25 M than for those in 0.5 M

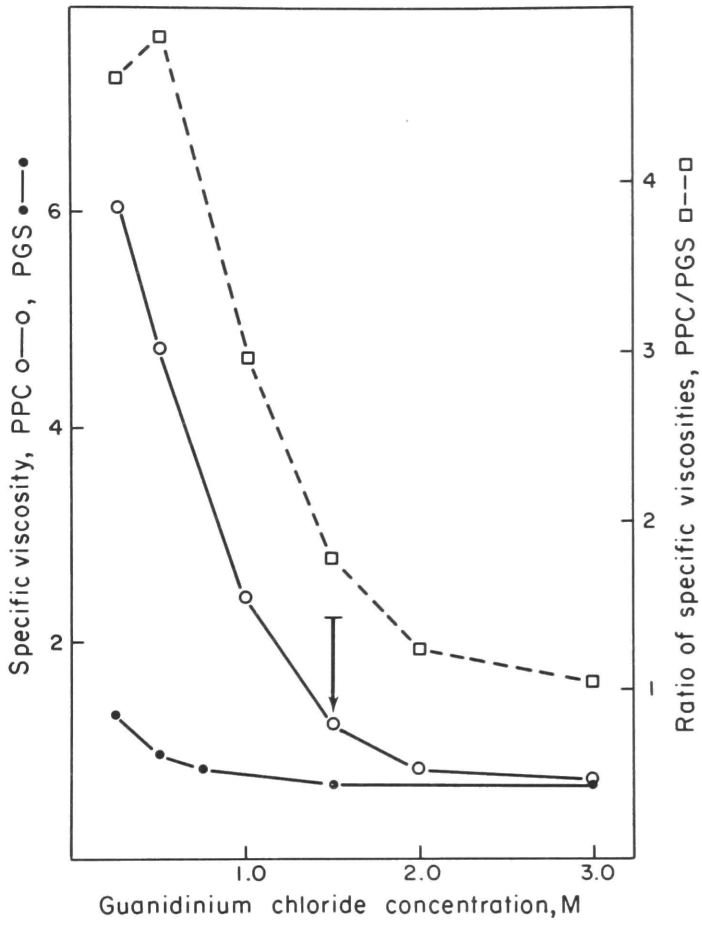


Figure 13. Disaggregation of PPC in high concentrations of guanidinium chloride. The specific viscosities of PPC (open circles) and of PGS (solid circles) were determined after the solutions were brought to the guanidinium chloride concentrations indicated on the abscissa as described in Experimental Procedure. The values for the PPC solutions 1.5 M or less in guanidinium chloride were evaluated 24 hours after the solutions were prepared; the arrow indicates the decrease in viscosity from one hour (bar) to 24 hours for the PPC solution at 1.5 M. The open boxes show the ratios of the specific viscosities of PPC to PGS. Solute concentrations were 0.6% (w/v).

guanidinium chloride. The viscosities of the PPC and PGS solutions are the same for concentrations of guanidinium chloride 2 M or greater, which indicates that aggregate is dissociated under these conditions. Other electrolytes, such as 3 M $MgCl_2$, also dissociate PPC (Sajdera and Hascall, 1969). These data are consistent with the hypothesis that interactions between PGS and GPL are in part ionic.

The viscosities of PPC solutions which were brought to intermediate electrolyte concentrations, 0.75 and 1.5 M in guanidinium chloride, decreased with time. The arrow in Figure 13 shows the change in viscosity between 1 hour (the bar) and 24 hours after bringing PPC to 1.5 M in guanidinium chloride. No changes in viscosity during the same amount of time were observed for any of the PGS preparations. These data offer a plausible explanation for the decreases of viscosity of protein-polysaccharide solutions with time that were observed by Malawista and Schubert, 1958, and more recently by Eyring and Yang, 1968. Their preparations probably contained significant amounts of aggregate which slowly dissociated in solution.

The viscosities of the PGS solutions become dependent upon electrolyte concentration below 0.5 M. This is indicated by the increase in the specific viscosity of PGS below this guanidinium chloride concentration. The increase is not due to aggregation, but reflects electrostatic interactions of at least two possible types: first, interparticle repulsion between the polyelectrolytes; and second, the repulsions of charged sites within the macromolecules which lead to increased effective hydrodynamic volumes. These charge effects become even more pronounced at ionic strengths lower than 0.25 M. In addition to their influence on solute rheological properties, such charge effects contribute to boundary hypersharpening in the ultracentrifuge and affect the diffusion characteristics of the macromolecules. The results indicate that 0.5 M guanidinium chloride is a sufficient concentration of electrolyte to suppress these charge effects at solute concentrations of PGS less than 0.6% (w/v).

Guanidinium chloride is an effective protein denaturant as well as an electrolyte, and it is possible that this property contributes to its effectiveness in destabilizing aggregate. Two additional reagents which are good protein denaturants, sodium dodecylsulfate and urea, were tested to see if they could disaggregate PPC. When an aliquot of 2% sodium dodecyl sulfate in 0.5 M NaCl was added to an equal aliquot of PPC in 0.5 M NaCl, 0.05 M MES, pH 5.8, the viscosity of the resulting solution decreased to a value within 5% of that of a PGS solution under the same conditions by the end of 16 hours. Similar results were observed when an aliquot of 12 M urea in 0.5 M NaCl was added to an equal aliquot of PPC in 0.5 M NaCl, 0.05 M MES, pH 5.8. Therefore, both 1% sodium dodecylsulfate and 6 M urea disaggregate PPC. They do not dissociate aggregate as rapidly as guanidinium chloride, probably because they are not cationic; but the fact that they disaggregate implies that native protein conformations within the GPL, or the PGS macromolecules, or both are involved in the aggregation process.

4. Effect of reduction on aggregation

The reduction and alkylation experiments support the hypothesis that native protein conformations are involved in aggregation. They also indicate that at least two chemically distinct interactions exist between GPL and PGS.

a. Reduction and alkylation of PPC in an associative solvent -- Figure 14 shows the g(s) profile of PPC in 0.4 M guanidinium chloride, 0.05 M tris-HCl, pH 8.5, before (solid line) and after (dashed line) treatment with 10 mM dithiothreitol for 2 hours at 37°C. The aggregates in the untreated PPC were dissociated by the reducing reagent, and the shift in macromolecules from the aggregate peak to the subunit peak increased the concentration of subunit in the treated sample relative to that in the untreated solution. This caused the subunit peak to be displaced to lower sedimentation coefficients in the treated sample (see Figure 19 in the next chapter). When aliquots of PGS in either 0.4 M or 4 M guanidinium chloride buffered at pH 8.5 were treated in a similar

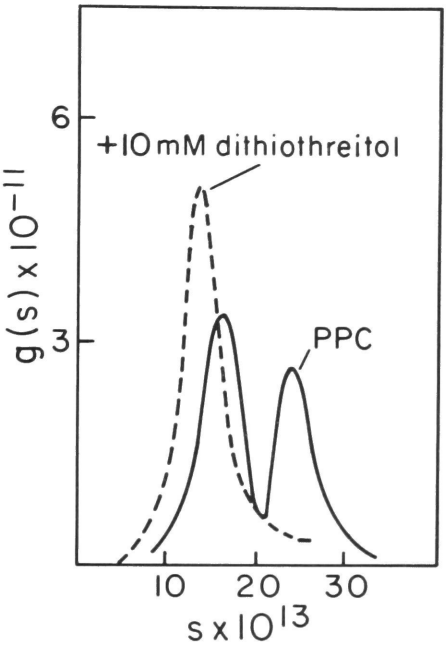


Figure 14. Disaggregation of PPC by treatment with dithiothreitol. The graph shows g(s) profiles of a PPC solution before (solid line) and after (dashed line) treatment with 10 mM dithiothreitol for 2 hours at 37°C. The solute concentration was 0.18%; the solvent was 0.4 M guanidinium chloride, 0.05 M tris-HCl, pH 8.5.

manner as the PPC preparation in this experiment, the g(s) profiles before and after exposure to dithiothreitol superimposed. This indicates that the subunit molecules are not further subdivided by the treatment with reducing reagent.

Amino acid analyses showed that about 80% of the cysteine residues were S-carboxymethylated by iodoacetamide after the sample was treated with dithiothreitol, while 0% were S-carboxymethylated by iodoacetamide without prior reduction. The results imply that the native protein structures of the macromolecules contain no free cysteine residues and that aggregation depends upon intact cystine disulfide bonds within the protein moieties. Presumably, by maintaining the conformations of the proteins, such intact disulfide bonds would be important in arranging the proper steric configurations of the chemical groups at the interaction sites.

Evidence presented in chapter IV shows that the aggregate contains about 13 PGS macromolecules. The geometry of organizing such a large number of proteoglycan subunits and glycoprotein links into an aggregate necessitates that both the GPL and PGS macromolecules have at least two physically separate interaction sites. If these sites are all identical, conditions which dissociate aggregate should be sufficient to physically separate GPL from PGS. If the sites are chemically distinct, conditions might be found which dissociate aggregate by reversing the interaction at one site, but which still leave GPL and PGS tightly associated through an interaction at another site that is not affected by the particular conditions of the experiment. The following experiment indicates that reduction of PPC, though sufficient to dissociate aggregate, does not separate GPL from PGS. This result supports the hypothesis that two or more distinct interaction sites exist.

PPC, which had been reduced and alkylated in 0.4 M guanidinium chloride, was centrifuged in a CsCl density gradient formed in the presence of 0.4 M guanidinium chloride. This gradient is similar to that used to prepare PPC, in which GPL and PGS are normally associated; and,

in this case, the GPL also co-sedimented with PGS. This is indicated in the upper half of Figure 15. No UV-absorbance was recovered in the upper fraction of the gradient; in fact, all the material in the gradient was recovered at the bottom. When the bottom fraction was redistributed in a CsCl gradient formed in the presence of 4 M guanidinium chloride, conditions which dissociate GPL from PGS, a component floated to the meniscus; this is shown in the bottom of Figure 15. Approximately 30% of the UV-absorbance is recovered in the top fraction, and there is a concomitant decrease in the ratio of UV-absorbance to hexuronic acid content in the bottom fraction as compared with the bottom fraction of the first gradient. GPL was associated with PGS in the first gradient, but dissociated from PGS in the second. Thus, conditions have been found which prevent aggregation (reduction with dithiothreitol) but which do not separate GPL and PGS under conditions in which they are normally associated (a density gradient formed in the presence of 0.4 M guanidinium chloride). This is good evidence that at least two chemically distinct GPL-PGS interactions exist. For convenience, the interactions will be discussed as occurring at site 1 (sensitive to reduction) and at site 2 (insensitive to reduction).

b. Reduction and alkylation in the presence of a dissociative solvent -- The previous experiment indicated that an interaction between GPL and PGS survived treatment with reducing reagent in an associative solvent where aggregate is already formed. It is possible that site 2 was protected under these conditions, and that if PPC were reduced when GPL and PGS are dissociated, interaction at site 2 might subsequently be prevented. To test this possibility, a sample of PPC was reduced and alkylated in 4 M guanidinium chloride, and then part of it was dialyzed against 0.4 M guanidinium chloride buffered at pH 8.5. A sample of the retentate was observed in the ultracentrifuge; and the g(s) analysis, shown in Figure 16, revealed only a single peak. Amino acid analyses indicated that about 90% of the total cystine content in the protein moieties of the macromolecules had been reduced and alkylated. CsCl density gradients were established for portions of the

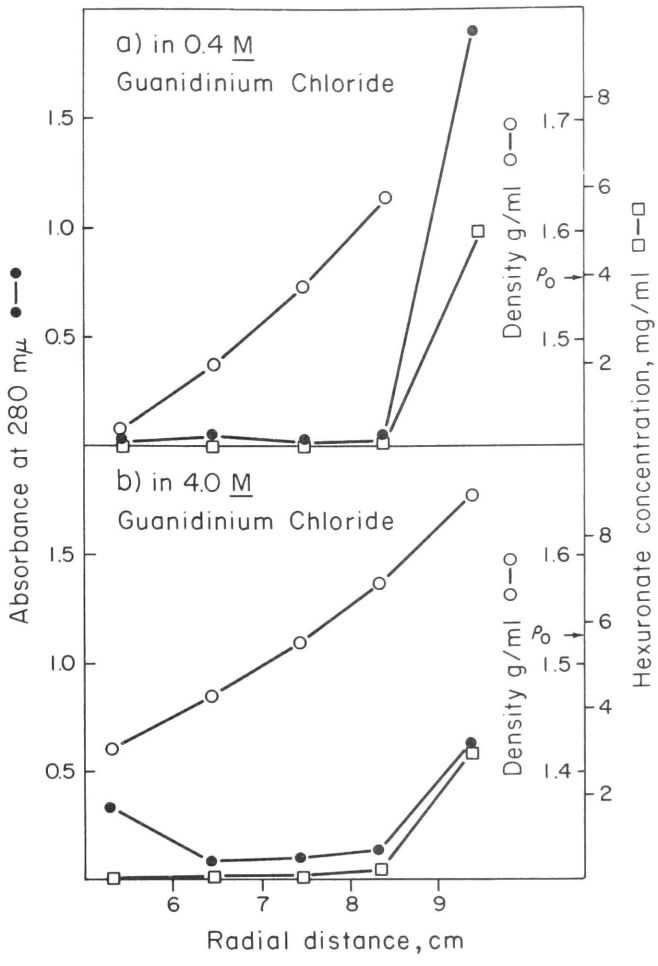


Figure 15. Evidence for an interaction of PGS and GPL which is unaffected by reduction of PPC.

a. The graphs show the equilibrium distributions of the absorbance at 280 mμ (solid circles) and of the hexuronic acid (open boxes) for reduced and alkylated PPC in a CsCl density gradient formed in the presence of 0.4 M guanidinium chloride.

b. The graphs show the equilibrium distributions of the absorbance at 280 mμ and of the hexuronic acid for the bottom fraction from the gradient pictured in part a in a CsCl density gradient formed in the presence of 4 M guanidinium chloride.

The open circles indicate the densities of the fractons; the ρ_0's indicate the initial densities of the solutions.

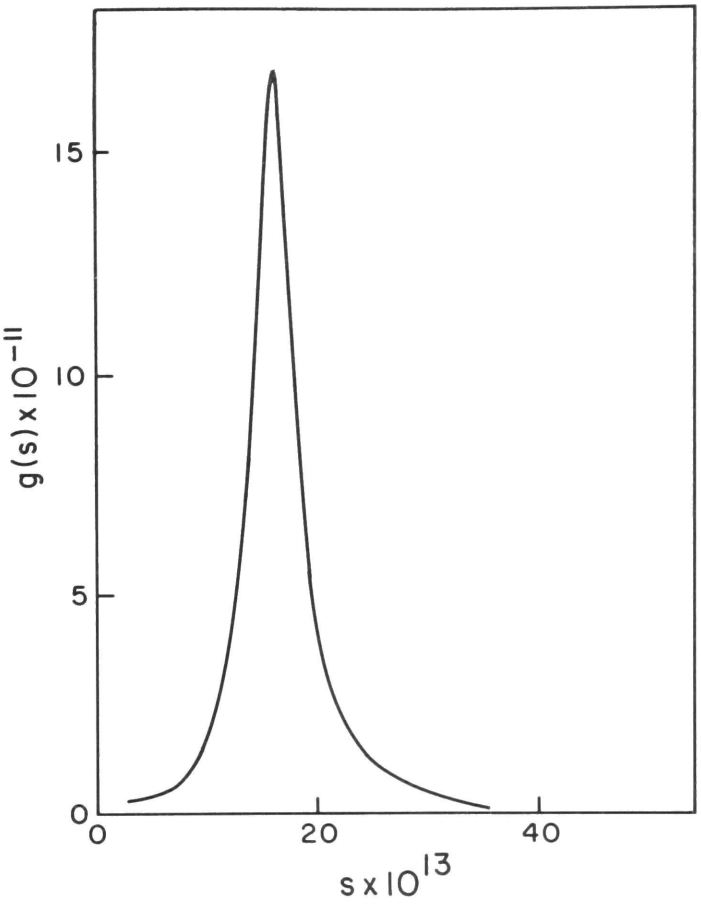


Figure 16. Sedimentation coefficient distribution, g(s), for reduced PPC. PPC was reduced and alkylated in 4 M guanidinium chloride as described in Experiment Procedure. The solute concentration for the centrifugal analysis was 0.15%; the solvent was 0.4 M guanidinium chloride, 0.05 M tris-HCl, pH 8.5.

reduced and alkylated PPC in 0.4 M and in 4 M guanidinium chloride. The upper fraction of the gradient formed in 0.4 M guanidinium chloride did not contain GPL, while that of the gradient formed in 4 M guanidinium chloride did. This is indicated by the absence of a peak of UV-absorbance at the top of the former gradient (upper half of Figure 17) and by the higher ratio of UV-absorbance to hexuronic acid found in the bottom fraction compared with that found in the bottom fraction of the latter gradient (lower half of Figure 17). It is apparent that GPL and PGS retain the capability of reinteracting at site 2 when they are dialyzed into an associative solvent even after the treatment with reducing reagent in dissociative solvent conditions.

Low solvent pH also fails to reverse this GPL-PGS interaction, even though such conditions dissociate aggregate (Figure 12 above). Aliquots of untreated PPC and of PPC that had been reduced and alkylated in 4 M guanidinium chloride were centrifuged in density gradients in 0.4 M guanidinium chloride buffered with formate at pH 2.5. In both cases, GPL still co-sedimented with PGS. To date, only 4 M guanidinium chloride has been shown definitely to reverse the interaction at the hypothetical site 2. The fact that it is not reversed by low pH or the ionic conditions of the density gradient in 0.4 M guanidinium chloride suggests that it may be mediated through interactions between hydrophobic regions within the protein moieties of the GPL and PGS molecules. If this is so, sodium dodecylsulfate may disaggregate PPC by reversing these interactions. Because sodium dodecylsulfate is not compatible with the CsCl density gradient methodology, other procedures, such as column chromatography, will have to be used to test this possibility.

c. Differential reduction of GPL and PGS -- The experiments described in parts a and b above were conducted at pH 8.5. These conditions were chosen because the reducing reagent becomes less effective in solvents with lower pH values. However, results described in Figure 11 above showed that the ability of PPC to form optimal amounts of

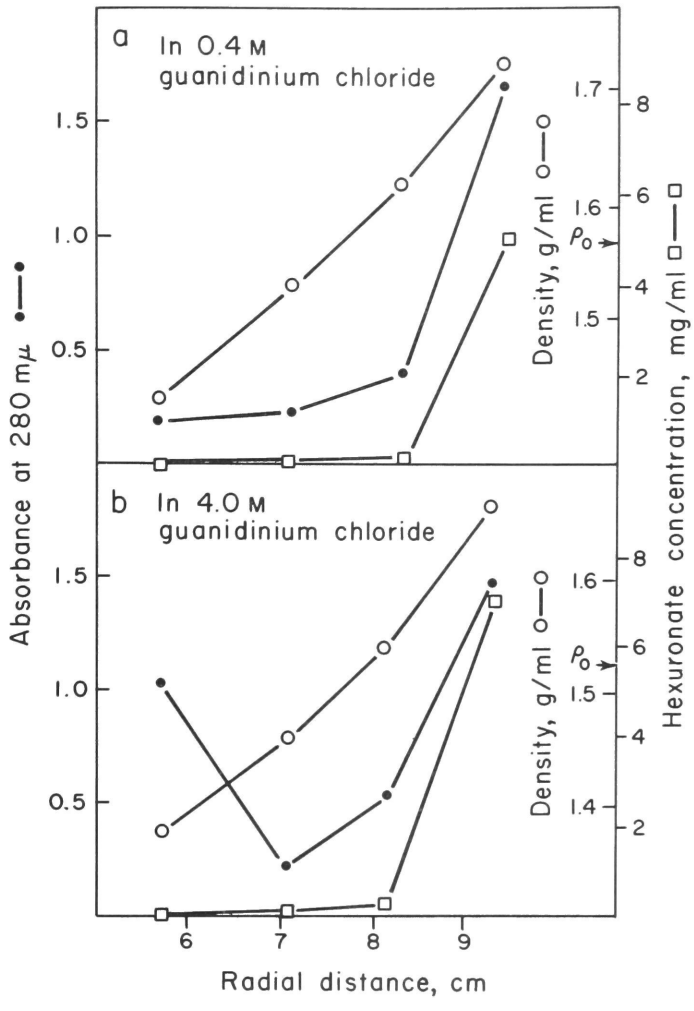


Figure 17. Evidence for the ability of reduced PGS and GPL to reassociate.

a. The graphs show the equilibrium distributions of the absorbance at 280 mμ (solid circles) and of the hexuronic acid (open boxes) for a PPC preparation that was reduced and alkylated in 4 M guanidinium chloride before dialysis against 0.4 M guanidinium chloride as described in Experimental Procedure. The CsCl density gradient was formed in the presence of 0.4 M guanidinium chloride.

b. The graphs show the equilibrium distributions of the absorbance at 280 mμ and the hexuronic acid for the same reduced and alkylated PPC in a CsCl density gradient formed in the presence of 4 M guanidinium chloride.

The open circles indicate the densities of the fractions; the ρ_0's indicate the initial densities of the solutions.

aggregate is adversely affected by exposure to pH 8.4 in dissociative solvent conditions. In order to design experiments to decide whether GPL, PGS, or both are sensitive to treatment with reducing reagents, it was first important to find conditions which allow reduction at pH 5.8, the optimal pH for recovery of aggregate.

The experiment shown in Figure 18a shows that the reducing reagent, dithiothreitol, does not dissociate aggregate in 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8. The addition of dithiothreitol to a concentration of 10 mM caused only a small decrease in the viscosity of a cartilage extract in this solvent (bar II); the decrease can be accounted for by sample dilution. Irreversible decreases in the solution viscosity occurred in the presence of the dithiothreitol only if the solution was heated. Bars III through V show the loss in viscosity after successive 10 minute treatments at 45°C, 54°C and 64°C. The viscosity after the last heat treatment is about that of an isolated PGS preparation under the same solvent conditions, which indicates that dissociation is essentially complete. The viscosity is considerably lower than that of a control aliquot which was heated at 64°C for 15 minutes in the absence of dithiothreitol (first shaded bar). This solution showed an increase in viscosity after the heat treatment over a period of 10 hours (indicated by the arrow). Roughly half the aggregate in the control survived the high temperature. When the extract was reduced in 3 M guanidinium chloride, 0.05 M MES, pH 5.8, before dialysis to 0.5 M guanidinium chloride, little or no aggregate was regenerated (second shaded bar). The sensitivity of aggregation to reduction with sulfhydryl reagents appears to be similar at pH 5.8 to that at pH 8.5, except that efficient reduction in this case has a strict dependence on 'forcing' conditions such as high temperature or dissociative solvent conditions. The results suggest that aggregates must be destabilized before reduction can proceed.

Because treatment with dithiothreitol at pH 5.8 was effective in preventing aggregation when reduction was carried out in 3 M guanidinium chloride, a dissociative solvent, the experiment shown in Figure 18b was

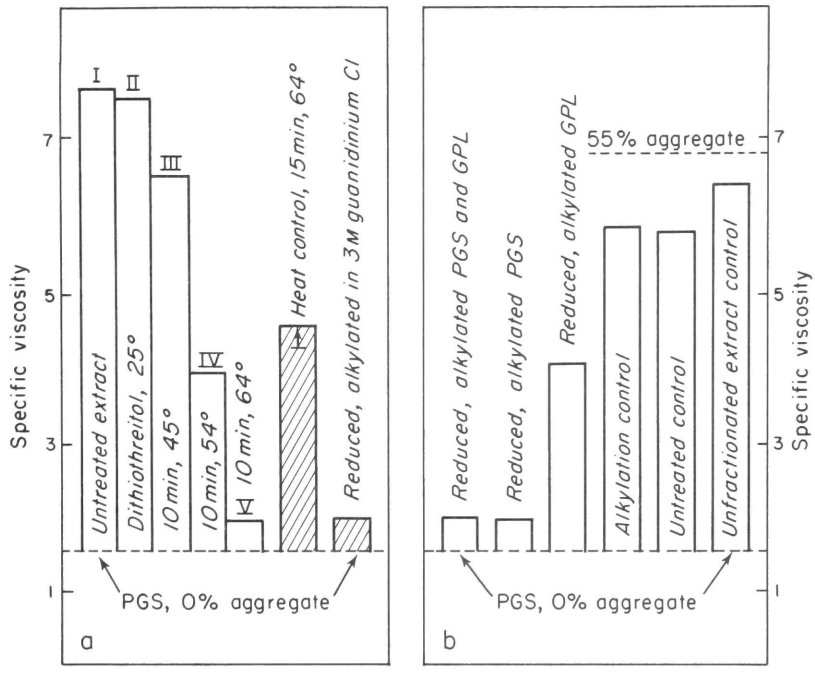


Figure 18. Effect of reduction and alkylation of PPC, PGS, and GPL at pH 5.8 on aggregation.

a. The bars represent the specific viscosity of a cartilage extract in 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8 (I); and of the extract after the sequential treatments: addition of dithiothreitol to 10 mM (II); heating for 10 minutes at 45°C (III), at 55°C (IV) and at 64°C (V). The first shaded bar shows the specific viscosity of an untreated extract which was heated for 15 minutes at 64°C; the arrow indicates the increase of viscosity during the 10 hours after heating. The second shaded bar shows the specific viscosity of an extract that was reduced and alkylated in 3 M guanidinium chloride at pH 5.8 before reaggregation. The solute concentrations were 0.45%.

b. The bars represent the specific viscosities of a series of mixtures of PGS and mixed glycoprotein which were prepared, treated and reaggregated as described in Experimental Procedure. The solutions were, from left to right: 1. both PGS and glycoprotein reduced and alkylated; 2. PGS reduced and alkylated, glycoprotein alkylated only; 3, PGS alkylated only, glycoprotein reduced and alkylated; 4. Both alkylated only; 5. Both untreated; 6. unfractionated extract. The upper dashed line is the specific viscosity of a PPC solution which had 55% aggregate. Solute concentrations were 0.45%.

done in order to test the sulfhydryl sensitivity of separated PGS and GPL. Aliquots of PGS and a mixed glycoprotein fraction were reduced and alkylated separately in 4 M guanidinium chloride at pH 5.8 as described in Experimental Procedure. When either untreated PGS and GPL, or alkylated but not reduced PGS and GPL were combined in a ratio comparable to that present in unfractionated extract and then reaggregated by dialysis into 0.5 M guanidinium chloride, the resulting solutions had specific viscosities which were only slightly lower than that of the unfractionated extract control. This indicates that the alkylating reagent alone had no affect on the reaggregation. Reduced and alkylated PGS combined with untreated GPL allowed little, if any, reaggregation; the specific viscosity of this mixture was as low as that observed for a mixture of PGS and GPL, both of which were reduced and alkylated. Neither solution had a specific viscosity significantly higher than a PGS solution with the same hexuronic acid concentration. Reduction and alkylation of GPL alone, however, was much less effective in preventing aggregation. When reduced and alkylated GPL was mixed with untreated PGS and the mixture reaggregated, it had a specific viscosity intermediate to that of reduced and alkylated PGS on the one hand and the alkylation control on the other. The GPL molecules are apparently affected by the reducing reagent, but it is probable that they are more resistant to reduction than the PGS molecules, and that the conditions chosen were inadequate to reduce them completely.

5. General discussion and conclusions

Mathews and Lozaityte, 1958, described some results which are clarified by observations described in this chapter. They found that 8 M urea and 2 M potassium thiocyanate reduced the viscosity of cartilage extracts which contained very high molecular weight proteinpolysaccharides (24-50 million as estimated from light scattering measurements). The viscosity of the solutions subsequently increased when these reagents were removed by dialysis. Also, a short exposure to 100°C irreversibly reduced the viscosity of the preparation. The authors suggested that their results were consistent with a model for proteinpolysaccharides

in which the molecules were aggregated and that those treatments which reduced the viscosity of their samples caused disaggregation. This is consistent with the interpretation of the experiments described in this chapter, and it is somewhat surprising that the implications of their results have, to a large extent, been overlooked.

Aggregation results from interactions between GPL and PGS macromolecules, and there are at least two distinct types of GPL-PGS interactions. The first, at hypothetical site 1, is sensitive to reduction and to low solvent pH and apparently involves some ionic associations between chemical groups within the two macromolecular components. The second interaction, at hypothetical site 2, is unaffected by these treatments. Both interaction are reversed by 4 M guanidinium chloride.

The interaction at site 2 has characteristics which indicate that it may involve hydrophobic regions within the macromolecules, which suggests that it involves protein to protein associations. On the other hand, the interaction at site 1 could involve an association between protein and polysaccharide because chondroitin sulfate has a carboxyl group in its structure which is titratable in the pH range that dissociates aggregate. However, the spatial geometry of the polysaccharide would have to be controlled by nearby protein conformations, since the interaction is reversed by treatment with sulfhydryl reagents. In addition, Mathews and Lozaityte, 1958, described experiments which provide some evidence that the chondroitin sulfate chains in the macromolecules are, in fact, not involved in aggregation. They determined the molecular weight of a proteinpolysaccharide preparation with light scattering measurements. The untreated material had an average molecular weight of about 24 million; in 2 M potassium thiocyanate, conditions where the macromolecules were dissociated, the molecular weight was only about 4 million. Therefore, the preparation contained a significant amount of aggregate. A sample of the untreated material was then digested with hyaluronidase to the extent that 85% of the chondroitin sulfate was removed from the macromolecules. The molecular weight of

the solution only fell to 9 million during the enzymatic treatment, a value which was considerably larger than the molecular weight of the subunit. This suggests that the aggregate complexes, though stripped of most of their chondroitin sulfate, did not dissociate, and that the GPL-PGS interactions were still intact. Thus, the acidic groups involved in the interaction at site 1 are probably within the protein portions of the macromolecules.

The experiments discussed in this chapter prove that the first GPL-PGS interaction is essential for aggregation; whenever it is reversed the aggregates dissociate. The experiments do not prove that the second one is also essential, but it is probable that subsequent experiments will document its role in the aggregation process. The nature of the interactions that hold GPL and PGS together and lead to aggregation *in vitro* apparently are not of the type proposed by Partridge, 1968, for the structure of the ground substance; the glycoprotein is not serving as a bridge between chondroitin sulfate chains on adjacent PGS macromolecules. It is still possible, however, that ionic interactions between polysaccharide and collagen or some other portion of the insoluble matrix, as suggested by Mathews, 1968b, are important for the cartilage structure *in vivo*. Such interactions would operate in addition to those described for PPC.

The presence of protein sites on PGS which interact with GPL would impose some restraints on the overall configuration of the PGS macromolecule. Protein comprises only about 7% of the total weight of PGS, and much of it must serve the structural function of covalently binding polysaccharide chains into the macromolecular structure. The interaction sites on the molecules would have to be physically accessible to the GPL binding sites; this requires that they be free of steric and electrostatic interference by the highly charged and bulky chondroitin sulfate chains.

The protein portion of proteinpolysaccharides has generally been assumed to be a featureless core, whose only function is to provide a

framework on which to covalently attach polysaccharide chains. Recently, Eyring and Yang, 1968, concluded from optical rotatory dispersion and circular dichroism measurements that the protein moiety consisted of "highly disordered protein (or proteins) with polysaccharide side chains attached to it." Disulfide and salt linkages between molecules were reported to be absent. The results presented in this chapter indicate that the protein portions of PGS and GPL are involved in the process of aggregation, and that their role in the structure of the ground substance needs to be re-evaluated.

Chapter IV

PHYSICAL PROPERTIES OF SUBUNIT AND AGGREGATE

A. Introduction

A number of investigations of the physical properties of nasal cartilage proteinpolysaccharides have been reported in the last 15 years. The methods selected for the studies have usually been the same, sedimentation velocity ultracentrifugation, viscometry and light scattering. However, the methods by which the proteinpolysaccharide samples were prepared were often different, and the results of the physical measurements have led to many different interpretations about the hydrodynamic characteristics of these macromolecules. Various models, which will be discussed in more detail in the general discussion, have been proposed. These include: 1) a rodlike basic molecular unit with a molecular weight of 4 million, a length of about 3700 Angstroms and an axial ratio of about 90 (Mathews and Lozaityte, 1958); 2) a polydisperse collection of unbranched coils with a molecular weight average of 2 million (Bernardi, 1957); 3) highly solvated macromolecules that occupy roughly spherical domains with average radii of gyration about 1400 Angstroms and molecular weights 3.2-5.8 million (Luscombe and Phelps, 1967a); 4) basic subunit molecular weights of 1.8 million (Eyring and Yang, 1968), or of 1 million (Schubert and Hamerman, 1968), or of 250,000 (Partridge, 1968).

The discrepancies in the measurements and interpretations of the physical properties of proteinpolysaccharides arise from several factors. Different isolation and purification procedures yield different amounts of the total proteinpolysaccharide in the tissue, and it has not been possible to define a representative proteinpolysaccharide preparation. Some shear degradation of the macromolecules occurs in the homogenization step which is often used to extract proteinpolysaccharides (Sajdera and Hascall, 1969). Isolated preparations have different amounts of aggregate and of non-covalently associated protein and collagen. Also, proteinpolysaccharides are polydisperse, and this fact has not been fully appreciated.

These problems can be circumvented by using the preparative methods described in the second chapter. The PGS fraction contains about 80% of the proteinpolysaccharide in the tissue and is representative of the bulk of the macromolecules. The extraction procedure does not introduce shear degradation, and it is unlikely that it disrupts any of the covalent structure of the macromolecules. PGS no longer contains aggregate or non-covalently associated protein or collagen. Also, the g(s) method for measuring the range of solute sedimentation coefficients allows a quantitative estimate of the extent of polydispersity of the macromolecules to be evaluated directly. For these reasons some of the physical properties of PGS were studied. Some measurements were also made for PPC in order to compare the physical characteristics of the aggregate with those of the subunit macromolecules.

The data indicate that PGS consists of a polydisperse class of macromolecules; 80% have molecular weights between about 1.3 and 4.1 million. The polydispersity results primarily from differences in the amount of chondroitin sulfate attached to the protein moieties. The PGS macromolecules have hydrodynamic characteristics which suggest that they are prolate ellipsoids with axial ratios of less than 4. They have very large associated solvent volumes, between 28-56 ml/g in 0.5 M guanidinium chloride. The aggregates in PPC contain an average of about 13 PGS molecules.

B. Experimental Procedure

1. Preparation of PPC and PGS

PPC and PGS were prepared in the Spinco 40 angle rotor as described in Experimental Procedure of chapter II. The samples were dialyzed against 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8, the solvent used for the following experiments.

2. Physical methods

a. Analytical ultracentrifugation -- Sedimentation velocity experiments were conducted for a number of PPC and PGS solutions with

different solute concentrations, 0.04-0.25% (w/v). The experimental conditions were those described in chapter II. Distributions of sedimentation coefficients, g(s), were computed for each concentration.

For a series of concentrations of PGS solutions, the integral form of the g(s) distribution function, G(s), was calculated for each finite concentration. The set of G(s) graphs was then extrapolated to zero concentration in order to estimate the thermodynamically ideal range of sedimentation coefficients exhibited by PGS for the conditions of the experiment, 0.5 M guanidinium chloride, 35,500 rpm, 20°C. The integral G(s) distribution and the extrapolation method are briefly described in the Appendix.

b. Viscometry -- Viscosity measurements were made on PPC and PGS solutions at different solute concentrations in an Ubbelohde dilution viscometer with four shear stresses. The value of the viscosity number, η_{sp}/c, was calculated for each solute concentration and shear stress. The specific viscosities, η_{sp}, were determined by the methods described in Experimental Procedure, chapter II; the concentrations in g/ml, c, were determined from the hexuronic acid concentration (measured as glucuronolactone) of the solutions and the measured amount of glucuronic acid in guanidinium-PGS, 25.7%. The values of the viscosity number were plotted as a function of concentration plus a constant times the shear stress in order to display the data in a plot analogous to that first presented by Zimm, 1948, for analyzing light scattering data (Figure 21 below). For each set of data, either PGS or PPC, the values for the viscosity number that were obtained at the 4 shear stresses were extrapolated to zero shear stress for each finite concentration; these extrapolated values yield a curve which shows the dependence of the viscosity on solute concentration in the absence of shear. The intrinsic viscosity, $[\eta]$, was determined by extrapolating this curve to zero concentration. In an analogous manner, the values for the viscosity numbers that were obtained at the different solute concentrations were extrapolated to zero concentration for each finite shear stress; these

extrapolated values yield a curve which indicates the dependence of the viscosity on shear stress in the absence of concentration effects. This curve provides information about the shape of the solute molecules. The details of this method for analyzing viscosity data are presented by Sajdera, 1969.

c. Packed volume of PPC and PGS -- A 1 ml aliquot of either PPC (0.24%) or of PGS (0.30%) in 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8, was loaded into a centrifuge cell equipped with a 12 mm Epon single-sectored centerpiece. 0.1 ml of fluorocarbon FC-43 was also loaded into the cell. The solution was then centrifuged in an ultracentrifuge at a series of rotor speeds between 22,000 rpm and 40,000 rpm. With time, at each rotor speed, the solute packed into a pellet on top of the fluorocarbon cushion at the bottom of the cell. Pictures of the schlieren patterns of the solution were taken at each rotor speed, and the radial positions of the air-solvent, solvent-pellet and pellet-fluorocarbon menisci were measured. Each rotor speed was maintained until the radial position of the solvent-pellet meniscus was the same over a four hour period. The ratio of the sectorial area of the pellet to that of the solvent plus pellet was calculated. This ratio and the concentration of solute were then used to calculate the specific volume in ml/g which the pellet occupied at each of the rotor speeds. After the value for the highest rotor speed was measured, the rotor was slowed to the speed at which the first measurement was made and the pellet volume redetermined. The values of the specific volume of the pellets for PPC and PGS were plotted against the reciprocal of the angular velocity squared, $1/\omega^2$, (Figure 22 below).

3. Partial fractionation of PGS

A PGS preparation (0.8%) was dialyzed against 4 M guanidinium chloride; CsCl was added to the retentate to give an initial density of 1.62 g/ml, (0.83 g CsCl per g of solution). A gradient was established by centrifugation at 26,000 rpm for 66 hours at 20°C in a Spinco SW 50.1 rotor. These conditions do not yield an equilibrium distribution of

the solute, but are such that the sedimenting solute boundary is spread throughout the CsCl gradient. The high guanidinium chloride concentration and the high density of the gradient partially fractionate the macromolecules on the basis of differences in buoyant densities and sedimentation velocities. Fractions were taken from the gradient and analyzed for density; they were then dialyzed against 0.5 M guanidinium chloride. The retentates were analyzed for absorbance at 280 mμ and for hexuronic acid. Aliquots of two fractions, fraction II from near the top of the gradient and fraction V from the bottom, were diluted to 0.20% (w/v) and were analyzed in the ultracentrifuge along with a sample of unfractionated PGS under the same conditions for comparison. Aliquots of both fractions, II and V, were dialyzed against 0.1 M KCl to remove guanidinium chloride and the hexosamine and amino acid contents of each were measured.

4. Treatment of proteinpolysaccharide with NaOH

The chondroitin sulfate chains are attached to the protein portion of proteinpolysaccharides through a glycosidic linkage to a serine hydroxyl in the polypeptide. This linkage is alkali labile and the β-elimination mechanism diagramed in Figure 4 above applies. Serines within the polypeptide which are not substituted are more resistant to treatment with base (Anderson, Hoffman and Meyer, 1965). Serines from which the polysaccharide chains are eliminated are transformed to dehydroalanine and are subsequently converted to pyruvic acid during acid hydrolysis; a net loss of serine can be observed. Thus, under appropriate conditions the number of alkali labile serines in a proteinpolysaccharide preparation can be used to estimate both the number of chondroitin sulfate chains per unit weight of protein of the macromolecules and the average number of disaccharide repeating units per chain. Many procedures have been reported for preparing chondroitin sulfate by treating cartilage or proteinpolysaccharide with alkali (Anderson *et. al.*, 1965, Marler and Davidson, 1965, Partridge and Elsden, 1961). The following experiments were designed to find the mildest conditions for the β-elimination reaction.

a. β-elimination as a function of NaOH concentration -- A proteinpolysaccharide fraction, PP-L3, was prepared from nasal cartilage by the method of Pal, Doganges and Schubert, 1966. It was isolated as the potassium salt and was dried over $CaCl_2$ *in vacuo*. These experiments were carried out before the dissociative extraction methods were discovered, which is why PP-L3 was used. However, the results are applicable to base elimination experiments with PGS fractions. Weighed samples of about 10 mg PP-L3 plus norleucine as an internal standard were treated in a pyrex test tube with 2 ml aliquots of solutions of NaOH (0.0 M to 1.0 M) at 2°C for 10 hours. Then 4 ml of 11.7 N HCl was added to each sample followed by a complementary 2 ml of NaOH (1.0 M to 0.0 M) in order that all samples would contain an equivalent amount of NaCl for the subsequent hydrolysis step. All the tubes were evacuated and sealed, and the samples were hydrolyzed at 110°C for 20 hours; the hydrolysates were flash dried. Amino acid analyses were made, and the micromoles of serine per mg of PP-L3 which survived treatment with the different concentrations of NaOH were calculated.

b. β-elimination as a function of time -- From the results in part a, a kinetic experiment was designed. 202 mg PP-L3 were dissolved in 10 ml of H_2O and chilled to 2°C; norleucine was included as an internal standard. At zero time, 10 ml of chilled 0.4 M NaOH was added. At later times, 1 ml aliquots were removed and added to 1 ml aliquots of 0.5 M acetic acid in pyrex tubes in order to acidify the mixtures and to stop the base elimination reaction. The tubes for each time point were capped and stored frozen until samples from all time points had been collected. Two ml of 11.7 N HCl was then added to all the samples, and the tubes were evacuated and sealed. The samples were hydrolyzed, flash dried and analyzed for amino acid content. The micromoles of serine per mg PP-L3 which survived the base treatment as a function of time were determined.

c. β-elimination of PGS fractions II and V -- The above experiments indicated that treatment of proteinpolysaccharide with 0.2 M NaOH

for 30 hours at 2°C is sufficient to destroy more than 95% of the serines within the macromolecules which are susceptible to β-elimination. These conditions were used on aliquots of fractions II and V prepared as described above in the partial fractionation of PGS; the samples were in 0.1 M KCl. After hydrolyses and amino acid analyses of base treated as well as untreated samples, the micromoles of serine lost after the treatment with base were calculated for each fraction.

C. Results and discussion

1. g(s) distributions of PGS and PPC at different solute concentrations

Figure 19 presents g(s) profiles calculated from centrifugal analyses of a number of different solute concentrations for PGS (upper) and PPC (lower). The slower sedimenting component of PPC and the single component of PGS exhibit the same characteristics with solute dilution. The single, fairly symmetrical peak spreads greatly at the lower concentrations. The large range of sedimentation coefficients exhibited by PGS (15-30 Svedbergs for the 0.05% (w/v) solution) results from a large amount of polydispersity in the subunit macromolecules. Diffusion of solute could occur in the course of a centrifugal experiment; this would contribute to boundary spreading and give a misleading estimate of the apparent solute polydispersity. However, the contribution of diffusion to the boundary spreading for PGS in these centrifugal experiments was shown to be negligible, as might be expected for large, bulky macromolecules (see Appendix). It is unlikely that the preparative procedure for PGS ruptures any covalent bonds within the macromolecules; and therefore, the amount of polydispersity of the preparation probably reflects that which is inherent for PGS in the tissue. The extent of the polydisperisty in terms of the molecular weights of the macromolecules is discussed below.

The aggregate component of PPC (shown by the dashed lines) comprises about 60% of the total weight of PPC. The aggregate peak exhibits much more pronounced concentration effects and has a greater degree of polydispersity than PGS. This would be expected because PGS, which

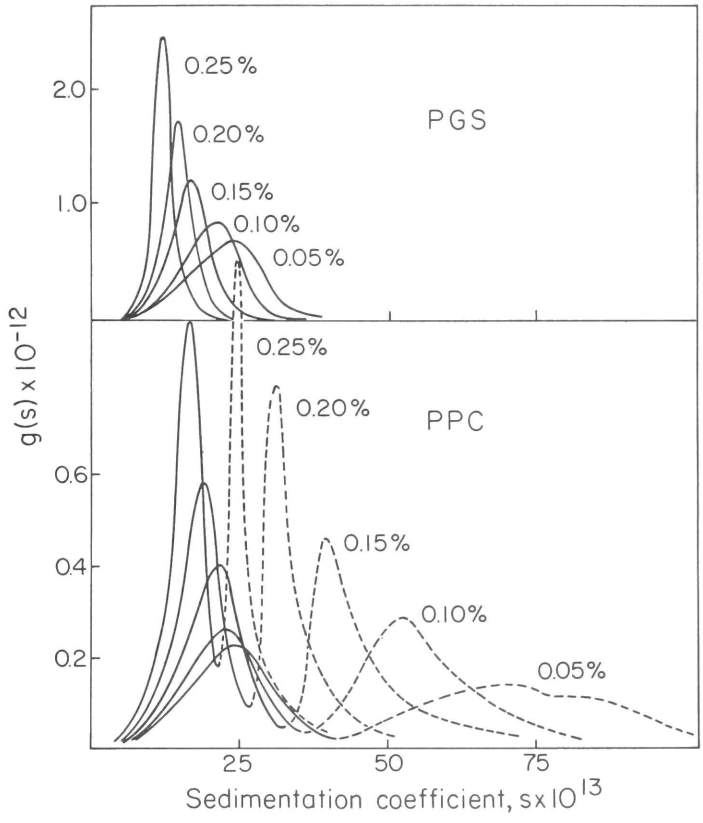


Figure 19. Centrifugal properties of PGS and PPC. The graphs show the g(s) profiles calculated from centrifugal analyses of PGS and PPC solutions at the different solute concentrations indicated. The peaks indicated by the solid lines in the PGS and PPC profiles consist of subunit macromolecules. The peaks indicated by the dashed lines in the PPC profiles consists of aggregates. The solvent was 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8.

constitutes all but a few percent of the weight of the aggregate, is polydisperse to begin with. It is possible that the average number of PGS molecules per aggregate lies within a fairly narrow range.

The sedimentation coefficients of the centers of the subunit and aggregate peaks of PPC at the different finite solute concentrations can be extrapolated to zero concentration. Figure 20 shows the reciprocal of these values (1/s) plotted against concentration for subunit (open boxes) and for aggregate (closed boxes). The values for a PP-L3 preparation (crosses), isolated by the procedures of Pal *et. al.*, are also shown on the graph and will be discussed later. The extrapolated values correspond to sedimentation coefficients of 26 Svedbergs and about 100 Svedbergs for subunit and aggregate respectively. These values represent thermodynamically ideal sedimentation coefficients for a hypothetical average subunit and aggregate. They can be used in conjunction with the intrinsic viscosities of PGS and PPC to estimate the number of subunits per aggregate as is discussed below.

2. Dependence of viscosity of PPC and PGS on concentration and shear

The outflow times of different concentrations of PPC and PGS in 0.5 M guanidinium chloride were measured in a dilution capillary viscometer at four different shear stresses. The values of the viscosity number, η_{sp}/c, were calculated for each concentration and shear stress. The data are presented in Figure 21, in which the viscosity number is plotted against the concentration in g/ml plus a constant times the shear stress. The graphs are analogous to those described by Zimm, 1948, for displaying light scattering data. This method for analyzing viscosity data was developed by Sajdera, 1969.

The large differences between the graphs for PPC and PGS result from the presence of 60% aggregate in the PPC preparation; and, in general, they demonstrate again how the presence of aggregate in a proteinpolysaccharide solution can greatly influence the physical properties of the solution. The curve through the open circles in each graph shows the shear dependence of the viscosity of PPC and PGS in the

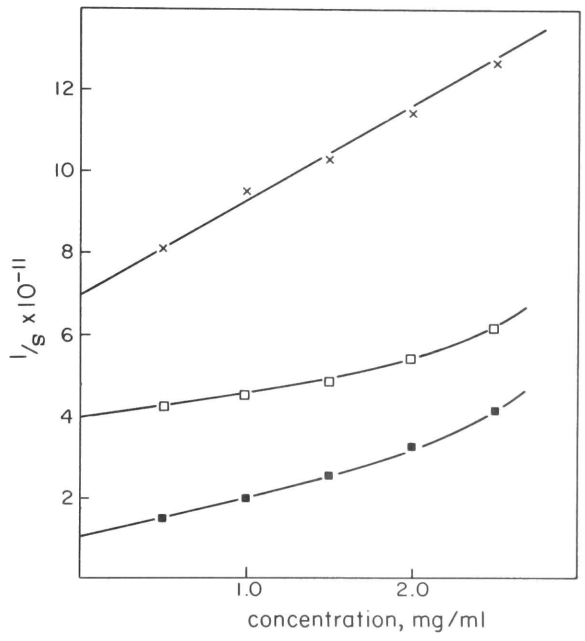


Figure 20. The graph plots the reciprocal of the sedimentation coefficients of the centers of the subunit peak (open boxes) and aggregate peak (closed boxes) of PPC against the solute concentration. A similar plot is shown for a PP-L3 preparation. The extrapolated values at zero concentration correspond to sedimentation coefficients of 26 Svedbergs for subunit, about 100 Svedbergs for aggregate, and 14 Svedbergs for PP-L3. The solvent was 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8.

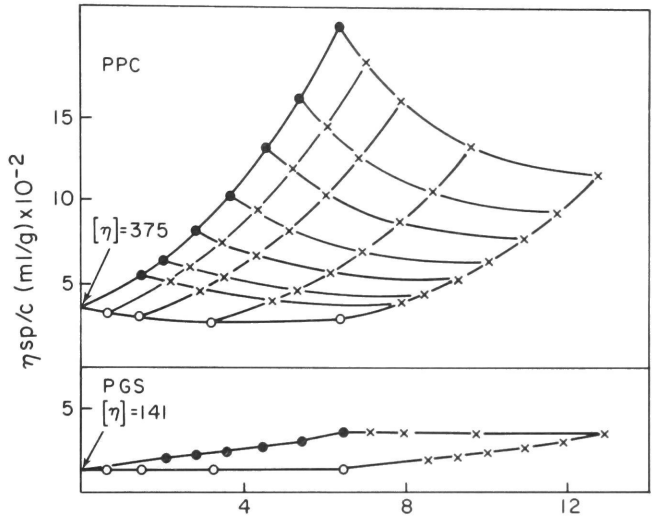


Figure 21. Dependence of the viscosity of PPC and PGS on concentration and shear stress. The values of the viscosity number, η_{sp}/c, (indicated by the X's) are plotted as a function of concentration plus a constant times the shear stress. Only the envelope values are indicated for PGS. The solid circles are the extrapolated values of the viscosity number at zero shear for each finite concentration. The open circles are the extrapolated values at zero concentration for each finite shear stress. The intrinsic viscosities, $[\eta]$, are the extrapolated values for the curves through the solid circles. The solvent was 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8.

absence of concentration effects; particle asymmetry should influence the shape of this curve. The viscosity of the aggregate complexes shows some shear dependence; this is indicated by the upswing in the curve for PPC as it approaches the ordinate. The viscosity of subunit shows almost no shear dependence; the curve for PGS is almost constant. These data suggest that the subunit is symmetrical and that the aggregate has a certain amount of asymmetry.

The curve through the solid circles in each graph shows the concentration dependence of the viscosity of each solute in the absence of shear effects. PPC shows a much larger concentration dependence than does PGS. This is consistent with an aggregate structure which is more asymmetric than subunit. An asymmetric aggregate would occupy a greater effective volume per g of dry weight in solution, and particle-particle interactions and electrostatic interactions would be increased at finite concentrations. The intrinsic viscosities, $[\eta]$, of the two solutions were calculated by extrapolating the concentration dependent curves (solid circles) to zero concentration. This gives a value of 141 ml/g for the subunit and 375 ml/g for PPC. If the contribution of the 40% subunit to the intrinsic viscosity of PPC is corrected for, the intrinsic viscosity of a solution which contained only aggregate would be about 560 ml/g. The fact that aggregate has a higher intrinsic viscosity than subunit is another indication that aggregate is more asymmetric. If both had the same axial ratio and contained the same amount of associated solvent, both should have the same intrinsic viscosity.

3. Estimation of the hydrodynamic volume of PGS and PPC

The value for the intrinsic viscosities of subunit and aggregate are large and indicate that the particles are either very asymmetric or have large volumes of associated solvent in solution. This section presents evidence which suggests that the latter possibility is more nearly correct.

The intrinsic viscosity for a particle depends upon two parameters, its shape and its effective hydrodynamic volume. The effective hydrodynamic volume of the particle in solution consists of two factors; first, the partial specific volume, $\bar{v}$, which is the volume that the dry weight of the particle occupies; and second, the volume of associated solvent, ϕ. This is shown in the equation at the upper left of Figure 22, where ν is the shape factor for the particle. For any value of the intrinsic viscosity for a solute, limits can be calculated in which the solute particles have either a maximum amount of associated solvent or have a maximum asymmetry. In the first case, the particle is assumed to be spherical; this defines the shape factor to be $\nu = 2.5$, (Einstein, 1906). The volume of associated solvent is then calculated. In the second case, the volume of associated solvent is assumed to be zero and a shape factor is calculated. This shape factor defines the axial ratio of an equivalent unhydrated prolate or oblate ellipsoid which would have the particular intrinsic viscosity, (Simha, 1940).

The limits of maximum solvation and maximum asymmetry were determined for PGS with $[\eta] = 141$ ml/g and $\bar{v} = 0.56$ ml/g (Figure 22, upper right). This value for $\bar{v}$ is essentially that which Eyring and Yang, 1968, used as the partial specific volume for a proteinpolysaccharide preparation in 5 M guanidinium chloride, 0.58 ml/g. The value used in this calculation, 0.56 ml/g, is corrected for the lower protein content of PGS as compared to their preparation. A spherical model for PGS predicts that the macromolecules would have an associated solvent volume of 56 ml/g. If the macromolecules contained no associated solvent, the calculated shape factor of 250 indicates that PGS would have an average axial asymmetry of 63 for a prolate ellipsoidal model. This is about the shape of a myosin molecule. An oblate ellipsoid with the same shape factor would have a much higher axial ratio. The viscosity of PGS was independent of shear (Figure 21 above) which suggests that the subunit macromolecules are not very asymmetric; therefore, the high intrinsic

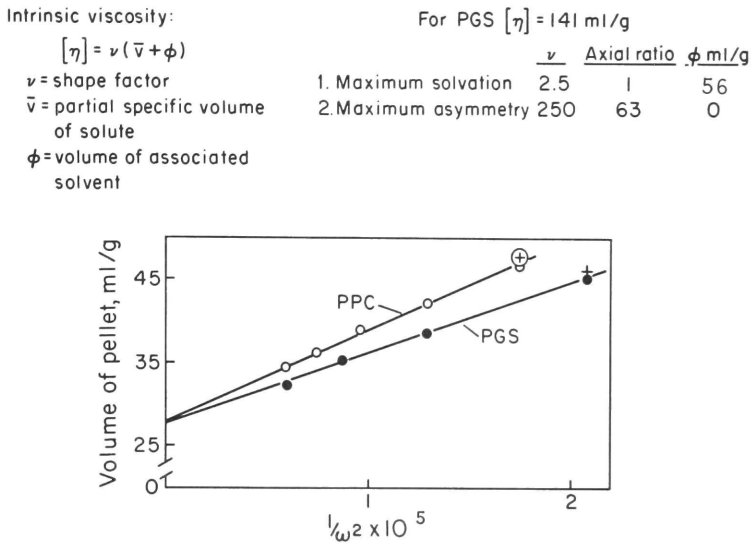


Figure 22. Estimation of the hydrodynamic volume of PGS. The equation in the upper left is the definition of the intrinsic viscosity. The calculations in the upper right indicate; 1. the maximum amount of solvent that can be associated with PGS or, 2. the maximum asymmetry for PGS, which are consistent with the equation for the intrinsic viscosity and the physical data for PGS; $[\eta]$ = 141 ml/g, $\bar{v}$ = 0.56 ml/g. The graph shows the packed volumes which PPC and PGS occupied when they were pelleted to the bottom of a centrifuge cell as described in Experimental Procedure; the values are plotted against one over the angular velocity squared, $1/\omega^2$, $(\text{radians/second})^{-2}$. The plus values indicate the volumes occupied by the solutes at the lowest rotor speeds at the end of the experiment; the other data at these points were determined at these rotor speeds at the beginning of the experiment.

viscosity implies that PGS macromolecules have a large volume of associated solvent.

The results of the experiment shown in the graph of Figure 22 provide additional evidence for this. A sample of PGS or of PPC was centrifuged in a single-sectored centerpiece along with a small amount of fluorocarbon FC-43. The fluorocarbon formed an inert cushion at the bottom of the cell and allowed the depth of the solute pellet that packed on top of it to be measured at a number of different rotor speeds. The calculated specific volumes of the pellet in ml/g at each rotor speed are plotted against the inverse of the angular velocity squared, $1/\omega^2$, which is proportional to the inverse of the pressure applied to the pellet. Extrapolation of this curve to zero is equivalent to extrapolation to infinite pressure, and the intercept is a rough estimate of the incompressible volume that the macromolecules occupy in these solvent conditions. The extrapolated value is about 28 ml/g for both PGS and PPC, which is good evidence that subunit and aggregate have about the same amount of associated solvent volume per dry weight. The incompressible volume should provide an estimate of the minimum hydrodynamic volume for the solute. A value of ϕ = 28 ml/g and an intrinsic viscosity of 141 ml/g correspond to a model for PGS of prolate ellipsoids with axial ratios of less than 4. Excluded volume is another estimate of molecular domain and can be used as a rough estimate of hydrodynamic volume. Gerber and Schubert, 1964, reported some results of equilibrium dialysis experiments of albumin in the presence of nasal cartilage proteinpolysaccharide. From their results in a number of solvents, they calculated an excluded volume for the macromolecules of 55 ml/g, a value which agrees well with the amount of associated volume predicted for PGS from its intrinsic viscosity if the macromolecules are spherical. The data, in sum, suggest that PGS macromolecules occupy roughly spherical domains in solution and have a large volume of associated solvent.

There are some other features about the pelleting experiment that are worth noting. The data indicate that it is more difficult to

compress aggregate than subunit (the curve for PPC has a greater slope), but that both have the same incompressible volume (both have the same intercept). This suggests that aggregate does not pack as efficiently as subunit, a result which may be a reflection of the greater asymmetry of aggregate complexes. The plus value in each graph is the value of the packed volume from the slowest rotor speed in each experiment; it was taken at the end of the experiment when the rotor was slowed down from the fastest speed. The points nearly coincide with those obtained at the same speeds initially. This indicates that the compressibility of the subunit and aggregate is reversible; this characteristic of the macromolecules may be important for the function they fulfil in the tissue. The value of the incompressible volume of the solute in this solvent is large enough so that in a 4% solution of PGS, the macromolecules in their hydrated state would fill all the available space in the solvent. Yet, in the tissue, the concentration of PGS is at least 15%. This means that the macromolecules in the cartilage matrix are either underhydrated or much of their charge is being neutralized by some component in the tissue. The observation of Mathews, 1968b, that chondroitin sulfate can form specific interactions with native collagen suggests that collagen might be involved in such charge neutralization.

The viscosimetric and centrifugal data which have been presented up to this point can be used to predict some possible hydrodynamic models for the aggregate complexes. The data presented in this section suggest that the subunit macromolecules are nearly spherical in solution and that subunits and aggregates have equivalent volumes of associated solvent per gram of dry weight; this implies that ϕ is about 56 ml/g for an aggregate complex. This value for ϕ and the value for the intrinsic viscosity of $[\eta]$ = 560 ml/g for an hypothetical solution of aggregates can be used to estimate the shape factor for an average aggregate complex by means of the equation for the intrinsic viscosity that is shown in Figure 22 above. The calculated shape factor corresponds to equivalent prolate and oblate ellipsoids with axial ratios of 8 and 13 respectively (Mehl, Oncley and Simha, 1940). These axial asymmetries can be

used in conjunction with other physical properties to estimate an average molecular weight for aggregate. Scheraga and Mandelkern, 1953, derived an equation which relates the intrinsic viscosities and sedimentation coefficients of prolate or oblate ellipsoids with their molecular weights.

$$M^{2/3} = Ns[\eta]^{1/3}\eta_0/\beta(1-\bar{v}\rho) \qquad (1)$$

where N is Avogadro's number; s is the ideal sedimentation coefficient, $[\eta]$ is the intrinsic viscosity (in deciliters/gram) and $\bar{v}$ is the partial specific volume of the solute; ρ is the density and η_0 is the viscosity (in stokes) of the solvent. β is a constant which depends upon the shape of the particle. For 0.5 M guanidinium chloride, ρ = 1.014 g/ml and η_0 = 0.01017 stokes. These values and those indicated in Table VI were substituted into equation 1, and average molecular weights of subunit and aggregate were calculated. The value of $\bar{v}$ was taken as 0.56 ml/g. Values for the molecular weight which are calculated from intrinsic viscosities and sedimentation coefficients are between the number average and the weight average molecular weights for disperse preparations (page 412, Tanford, 1961). Thus, the molecular weights listed in the table for subunit and aggregate are less than the weight average molecular weights. The ratio of the molecular weights of aggregate to subunit listed in the last column for prolate and oblate ellipsoids are estimates of the number of subunits which would be expected for an average aggregate with the indicated shapes. It is not possible to construct an oblate ellipsoid with an axial ratio of 13 from only 15 spherical subunits, and therefore this model for the aggregate complex is unlikely. However, it is possible to construct an aggregate with a staggered array of about 13 spherical subunits into a complex which behaves as if it were a prolate ellipsoid with an axial ratio of about 8; and therefore this model is consistent with the data presented above.

Overall, the centrifugal and viscosimetric data are consistent with the following model for subunit and aggregate. The subunit is a polydisperse collection of prolate ellipsoids with little or no axial asymmetry,

Table VI

Estimation of average molecular weights for subunit and aggregate from centrifugal and viscosimetric data

	[a] $s*10^{13}$	$[\eta]$ dl/g	axial ratio	[b] $\beta*10^{-6}$	[c] $M*10^{-6}$	$\frac{M_{agg}}{M_{sub}}$
subunit	26	1.41	1	2.12	2.7	
aggregate	100	5.60				
prolate			8	2.35	35.0	13
oblate			13	2.14	41.0	15

[a] The sedimentation coefficients are the centers of the peaks for subunit and aggregate shown in Figure 19 extrapolated to zero concentration.

[b] Values for β are taken from Scheraga and Mandelkern, 1953.

[c] Calculated from equation 1 in text.

and with an average molecular weight of about 2.7 million. The subunit macromolecules occupy large domains in solution and have associated solvent volumes in 0.5 M guanidinium chloride which may be as high as 56 ml/g. In the presence of glycoprotein link, an average number of about 13 subunits form an aggregate complex. The hydrodynamic properties of the aggregate are consistent with those of an equivalent prolate ellipsoid with an axial ratio of 8.

4. Estimation of the extent of molecular weight polydispersity of PGS

The Scheraga and Mandelkern equation and the g(s) method can be used to estimate the extent of polydispersity of the PGS macromolecules in terms of their molecular weights. When the value of the intrinsic viscosity of PGS is substituted into equation 1, the following relationship between M and s results:

$$M = 2.08(10^{17})s^{3/2} \qquad (2)$$

The primary assumption that has been made in calculating equation 2 is that the intrinsic viscosities for the PGS macromolecules are independent of their molecular weights. This would be true if all the macromolecules are spherical or have the same axial ratio, and if they have the same amounts of associated solvent per gram of dry weight. Data presented in the previous section support this assumption as does a result from the work of Pal, Doganges and Schubert, 1966. They reported an intrinsic viscosity of 140 ml/g for a proteinpolysaccharide preparation, PP-L3, in 0.1 M KCl. PP-L3 is purified by differential centrifugal techniques and contains macromolecules from the slowest sedimenting portion of the PGS distribution. The PP-L3 macromolecules are also partially shear degraded, and the preparation has an average sedimentation coefficient at infinite dilution of only 14 Svedbergs as is indicated in Figure 20 above. Thus, PP-L3 contains macromolecules which have lower average molecular weights than those in PGS, but both preparations have the same intrinsic viscosity.

Figure 23 shows the integral G(s) distributions that were calculated for PGS solutions at a number of solute concentrations. These curves were then extrapolated to zero concentration to give the thermodynamically ideal range of solute sedimentation coefficients for the macromolecules in 0.5 M guanidinium chloride (dashed line). The outside vertical lines correspond to G(s) = 0.1 and 0.9; they indicate that 80% of the weight of the PGS preparation sediments with velocities between 16 and 34 Svedbergs. The values for the molecular weights indicated for these limits on the bottom abscissa were determined from equation 2; they suggest that 80% of the weight of PGS resides in macromolecules with molecular weights between 1.3 and 4.1 million. The radii, R, for spherical particles with these molecular weights can be calculated from the volumes of the solvated particles:

$$V = (4\pi/3)R^3 = (M/N)(\bar{v}+\phi)$$

$$= (M/N)([\eta]/\nu) \qquad (3)$$

The values, indicated in Figure 23, range from 320 to 420 Angstroms. The extended length of an average chondroitin sulfate chain, which has about 40 repeating disaccharide units, is about 300 to 400 Angstroms. If the chondroitin sulfate chains radiate out from the protein core in the macromolecules, the length of the chains could be an estimate of the possible radii of the domains that the PGS species occupy; in this case the two values would be in good agreement.

Scheraga and Mandelkern, 1953, also showed that the relationship between intrinsic viscosity, sedimentation coefficient and molecular weight is relatively insensitive to the shape of prolate ellipsoids with axial ratios less than about 10, and a correction to equation 2 for axial ratios of less than 4 will not change the range of molecular weights predicted for PGS significantly. The value chosen for the partial specific volume, however, will influence the range of molecular weights. Equation 2 assumes that $\bar{v}$ is a constant for all macromolecules in the distribution. The next section indicates that this is an oversimplification because the amount of protein in the macromolecules

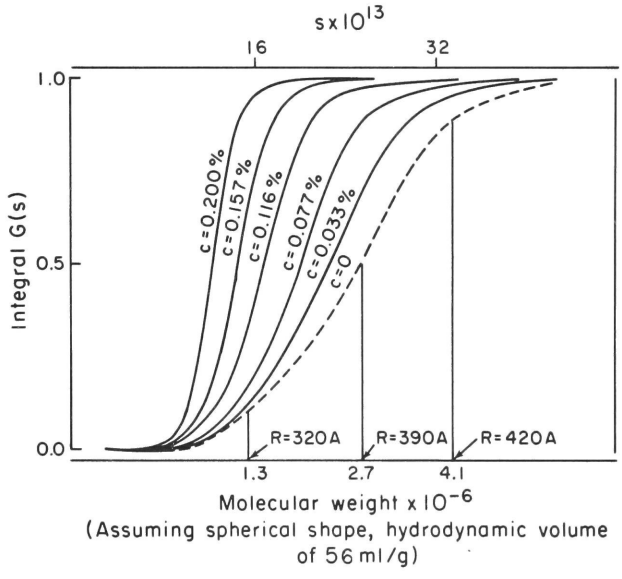


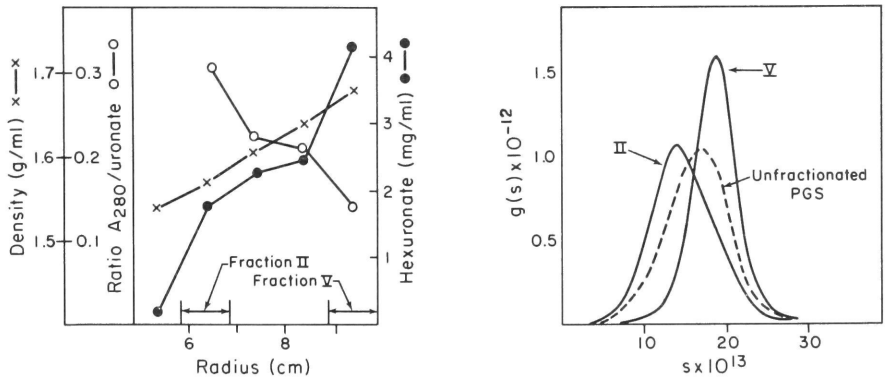
Figure 23. Extent of molecular weight polydispersity of PGS. Integral G(s) curves were calculated from centrifugal analyses for a number of different PGS solute concentrations; the set of curves was then extrapolated to zero concentration (dashed line). The sedimentation coefficients are indicated on the upper abscissa. The calculated molecular weights (equation 2, text) for the sedimentation coefficients which correspond to values of G(s) = 0.1, 0.5 and 0.9 are indicated on the lower abscissa along with the radii of equivalent spheres for these molecular weights (equation 3, text). The solvent was 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8.

is more than 10% for the lowest molecular weight species and less than 6% for those with highest molecular weights. These differences will introduce a variation in $\bar{v}$ because the protein and polysaccharide components in the macromolecules have different partial specific volumes. If corrections are made for this amount of variation, the distribution of molecular weights would be slightly smaller, 1.4-4.0 million, than the range predicted on the basis of constant $\bar{v}$. The absolute value chosen for $\bar{v}$ was that suggested by Eyring and Yang for proteinpolysaccharide in 5 M guanidinium chloride. Preliminary measurements of the partial specific volume of the guanidinium salt of PGS in 0.5 M guanidinium chloride indicate that the value 0.56 ml/g is probably too low and that $\bar{v}$ may, in fact, be as high as 0.62 ml/g. If this value is taken as an upper limit and is substituted into equation 2 the molecular weight range would be shifted to higher values, 1.6-5.1 million. Clearly, the most important problem is to determine a precise value of the average $\bar{v}$ for PGS in 0.5 M guanidinium chloride because imprecision in this value can introduce a large error. Such experiments have not been completed.

5. Partial molecular weight fractionation of PGS

The data presented in the previous section indicated that the PGS macromolecules are polydisperse with a very wide range of molecular weights. The experiment presented in this section shows that the primary compositional characteristic responsible for this polydispersity is that the macromolecules have differences in the proportion of chondroitin sulfate to protein each contains.

The CsCl gradient described on the left of Figure 24 was established at high density and in the presence of 4 M guanidinium chloride. The guanidinium salt of PGS has a lower buoyant density than that of the cesium salt, and guanidinium competition for the anionic groups in the macromolecules in the mixed gradient tends to lower the buoyant densities of the macromolecules. The lower solute buoyant densities and higher absolute densities in the gradient slow the sedimentation



	Polypeptide	GlcN/GalN
Fraction II	10.4%	0.12
PGS	6.8%	0.083
Fraction V	5.5%	0.054

Figure 24. Partial fractionation of PGS by density gradient centrifugation. The graphs at the left show the distribution of hexuronic acid (solid circles) and the ratio of absorbance at 280 mμ to hexuronic acid (open circles) for PGS in a CsCl density gradient established under the conditions indicated. The densities of the fractions are indicated by the X's. Fractions II and V were analyzed in the ultracentrifuge; their g(s) profiles are presented at the right along with one for PGS (dashed line) for comparison. The solute concentrations were 0.20%; the solvent was 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8. The table at the lower right shows the percentage of polypeptide in the fractions (calculated from the sum of the amino acid residues and the hexuronic acid content in each), and the ratios of glucosamine to galactosamine in each.

velocities of the macromolecules into the gradient. As it turns out, those macromolecules with the fastest sedimentation velocities, and hence with the largest molecular weights, also have higher densities. These two factors combine to spread the PGS distribution through the gradient in such a way that the bottom fractions contain greater percentages of macromolecules with higher molecular weights. The distribution of chondroitin sulfate in the gradient is indicated by the hexuronic acid content of the fractions (solid circles). If the gradient were centrifuged longer, this distribution of polysaccharide would sediment further into the gradient. This indicates that the gradient was not at equilibrium with respect to solute. The amount of absorbance at 280 mμ per hexuronic acid content in each fraction decreased across the gradient (open circles) which suggests that the gradient is partitioning the molecules on the basis of chondroitin sulfate to protein ratios. This was confirmed when physical and chemical analyses of fraction II were contrasted with those of fraction V. Samples of fraction II and fraction V at identical solute concentrations were analyzed in the ultracentrifuge, and g(s) distributions were calculated. The profiles, along with one for a sample of unfractionated PGS, are presented in the right half of Figure 24. The molecules in fraction II have lower average sedimentation coefficients than those in fraction V, and thus they have lower average molecular weights. The table in the lower right of Figure 24 shows that fraction II has a greater percentage of polypeptide than fraction V, which indicates that it has less chondroitin sulfate per protein. The ratio of glucosamine to galactosamine in each fraction correlates with the polypeptide content in each, which suggests that the amount of keratan sulfate parallels the amount of protein in the fractions. Partial amino acid compositions for the two fractions are presented in the first two columns of Table VII. None of the amino acids differed significantly between the two fractions with the possible exception of serine, which was 10% higher in fraction V.

The results suggest the following model for the macromolecules in the PGS preparation: the molecules have a protein core of a particular size and amino acid composition; the amount of chondroitin sulfate attached to the protein varies from molecule to molecule, whereas the amount of keratan

Table VII

Partial amino acid composition[a] of fractions II and V before and after treatment with base

	residues per 843 residues[b]			
	fraction II	fraction V	fraction II base treated[c]	fraction V base treated[c]
Serine	114	127	69	65
Aspartic acid	70	69	74	72
Threonine	66	68	64	66
Glutamic acid	150	142	153	148
Proline	104	101	109	98
Glycine	112	117	108	120
Alanine	73	67	70	68
Valine	60	60	61	64
Isoleucine	28	27	29	29
Leucine	63	67	63	67

[a] Basic amino acids were not measured; the glucosamine peak chromatographed in the region of the aromatic amino acids and prevented an accurate determination of tyrosine and phenylalanine in this experiment.

[b] The data for PGS in Table V indicate that the amino acids presented in this table account for about 843 residues per 1000 residues in PGS.

[c] 0.2 M NaOH, 2° C, 30 hours.

sulfate incorporated into each is relatively constant. The molecular weight range of 1.3-4.1 million estimated for PGS in the last section, suggests that those molecules with the highest molecular weights should have 3-4 times more chondroitin sulfate per protein as those with the lowest molecular weights to be consistent with the model. Although the centrifugal results presented in Figure 24 indicate that the g(s) profiles for fractions II and V overlapped considerably, fraction V nevertheless had a ratio of hexuronic acid to protein which was about 1.65 times that for fraction II.* This ratio would undoubtably be larger if the fractionation procedure were more efficient, and the data are consistent with the expected range of molecular weights.

6. β-elimination reaction of substituted serines in proteinpolysaccharide

The variation in the amount of chondroitin sulfate per protein, which is the primary reason for the polydispersity of PGS, could result either from different numbers of polysaccharide chains attached to the protein moiety, or from different total lengths for the individual chains, or from some combination of both factors. Theoretically it is possible to distinguish the relative importance of these two possibilities by determining the total number of attachment sites between protein and polysaccharide for macromolecules from the distribution which have different molecular weights. If only the number of chains per molecule were different, the number of attachment sites would be greater for the molecules with higher molecular weights. If only the lengths of the chains differed, the number of attachment sites would be the same for all molecular weight species. Two things are required

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* The ratio of the micromoles of each amino acid per micromoles of hexuronic acid, AA_i/HA, was calculated for amino acids in both fractions II and V. If the amino acid composition of the protein in each fraction is identical, the ratio:

$$X = \left[AA_i(II)/HA(II)\right] \Big/ \left[AA_i(V)/HA(V)\right]$$

will be the same for all amino acids and will provide an estimate of the relative amount of hexuronic acid (and hence of chondroitin sulfate) in fraction V as compared with fraction II. The value of X for the amino acids listed in Table V was found to be 1.63 ± 0.08.

to test these possibilities; first, an assay for the number of attachment sites, and second, a method for preparing PGS fractions which contain different populations of molecular weights. The serines to which the chondroitin sulfate chains are attached to the protein in PGS are susceptible to the β-elimination reaction described in Figure 4 above, and the loss of serine after treatment of proteinpolysaccharide with dilute alkali offers a reasonable chance for assaying the number of attachment sites. In the fractions of PGS that were described in the previous section, PGS was partitioned on the basis of solute molecular weights; fractions II and V were biased toward low and high molecular weight species respectively. For these reasons, attempts have been made to measure the number of serines within the protein moieties of fractions II and V which undergo β-elimination when treated with NaOH.

Sufficient conditions for optimizing the β-elimination reaction without causing destruction of unsubstituted serine were determined in earlier experiments on a proteinpolysaccharide, PP-L3, prepared by the methods of Pal, Doganges and Schubert, 1966; the results of these experiments are shown in Figure 25. The upper graph shows the loss of serine which occurred when PP-L3 was treated with different concentrations of NaOH for 10 hours at 2° C. Increasing the base concentration from 0.5 M to 1.0 M did not cause much additional loss of serine in this time period. The lower graph indicates the loss of serine in PP-L3 as a function of time in 0.2 M NaOH, 2° C. The net decrease in serine recovery reached a maximum by 30 hours; about 60% of the total serines were destroyed. No further loss occurred during the next 3 days. The number of micromoles of uronic acid per mg of PP-L3 divided by the number of micromoles of serine lost per mg of PP-L3 is an estimate of the number average of disaccharide repeating units for the chondroitin sulfate chains; in this case n = 31.5, which would correspond to a molecular weight for the guanidinium salt of chondroitin sulfate of M_n = 18,300. The contents of the other amino acids and of hexuronic acid in PP-L3 were not affected by exposure to the base treatment in either of the two experiments.

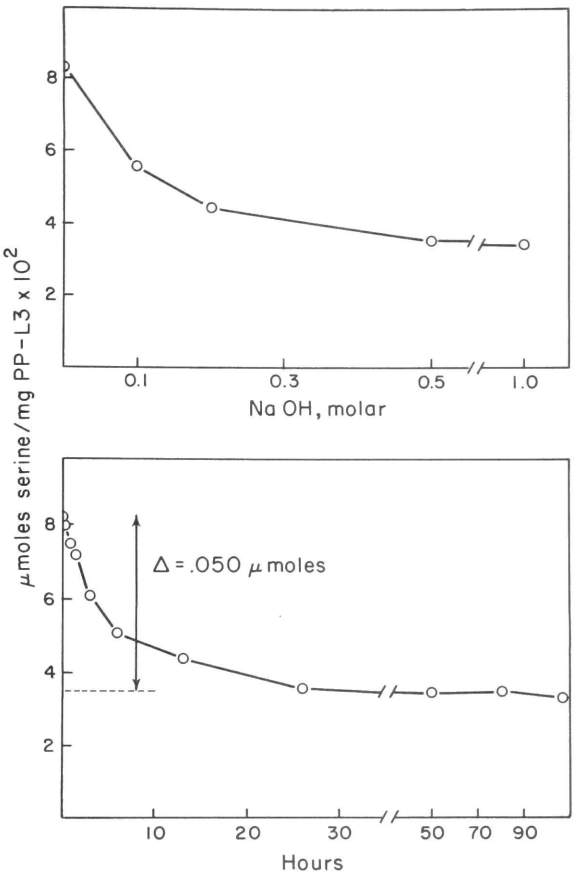

Figure 25. β-elimination of chondroitin sulfate chains from serines in a proteinpolysaccharide preparation, PP-L3. The upper graph indicates the amount of serine lost when PP-L3 was treated for 10 hours at 2°C with the concentration of base indicated by the abscissa. The lower graph indicates the amount of serine lost as a function of time when PP-L3 was treated with 0.2 M NaOH at 2°C. The total decrease, Δ, of 0.050 micromoles serine per mg PP-L3 corresponds to an average chondroitin sulfate chain length with 31.5 repeating disaccharide units.

The results of the experiments with PP-L3 indicate that treatment of a proteinpolysaccharide preparation with 0.2 M NaOH for 30 hours at 2° C is sufficient to destroy essentially all the substituted serines. These conditions were employed on fractions II and V obtained from the gradient described in the previous section, and amino acid analyses were made. The partial amino acid compositions are shown in columns 3 and 4 of Table VII above. Only serine showed significant differences when the results were compared with the analyses of the untreated samples shown in the same table. The data indicate that fraction II, which contains macromolecules with lower average molecular weights, lost 39% of its total serine content. This corresponds to an average number of disaccharide repeat units, n, of 37.5 and a number average chain molecular weight, M_n, of 21,600 for the guanidinium salt of chondroitin sulfate. Fraction V which contains macromolecules with higher average molecular weights, lost 49% of its total serine content; this gives $n = 43.5$ and $M_n = 25,200$. These results imply that differences in both the number of chains and in chain lengths contribute to the polydispersity of the PGS macromolecules. Fewer serines appear to be substituted and the average chain lengths appear to be less in fraction II than in fraction V.

Two factors can influence these results. First, it is possible that the loss of serine is not a true reflection of the number of chondroitin sulfate chains in the macromolecules; for instance, some of the chains could be attached to other linkages, serines could be substituted with other groups which eliminate in base, or elimination could be inefficient. Secondly, losses in serine may occur in the acid hydrolysis conditions used to prepare samples for amino acid analysis.

The data presented by Anderson, Hoffman and Meyer, 1965, indicate that serine hydroxyls are the major, if not only, linkage between protein and chondroitin-4-sulfate in nasal cartilage. They also showed that unsubstituted serines were not affected by base treatment; in somewhat harsher conditions, 0.5 M NaOH, 20° C, 19 hours, neither edestin nor α1-glycoprotein lost significant amounts of serine. There are no other groups which are known to be serine-O-substituted in nasal cartilage

proteinpolysaccharides. If such groups exist, it is unlikely that they comprise a significant fraction of the total because the molecular weights estimated for the chondroitin sulfate chains from the base elimination results are close to those determined in several laboratories for chondroitin sulfate which was prepared by treatment of proteinpolysaccharide with papain: 28,000 (Mathews, 1956, Partridge, Davis and Adair, 1961), 22,000 (Buddecke, Kröz and Lanka, 1963) and 21,000 (Luscombe and Phelps, 1967b). Sogn, 1968, used CsCl density gradients to separate protein from chondroitin sulfate after PGS was treated with base under identical conditions as those used in this experiment. About 80% of the protein was recovered with no detectable chondroitin sulfate attached. This indicates that essentially all of the chondroitin sulfate chains in most macromolecules were removed from the protein moieties, which suggests that the elimination reaction is efficient. The results, in sum, support the hypothesis that the loss of serine is a good indication of the number of chondroitin sulfate chains attached to the protein. The recovery of serine in proteinpolysaccharide hydrolysates as a function of hydrolysis time or of hydrolysis conditions has not been studied. Hirs, Stein and Moore, 1954, reported that 16% of the serines in ribonuclease were destroyed by hydrolysis under similar conditions as were used in this experiment. If serine is destroyed to the same extent in the PGS fractions, the values of n and M_n would be decreased 31.5 and 18,200 for fraction II, and to 36.5 and 21,100 for fraction V. Qualitatively, however, the results would be the same.

There is an additional problem which indicates that the results of the base elimination experiment should be viewed with caution. The product of the percentage of substituted serines multiplied by the average nolecular weight for the chondroitin sulfate chains in fraction V (49 X 25,200) can be compared to that for fraction II (39 X 21,600). The ratio of the two values is approximately 1.45, and it is an estimate of how much more chondroitin sulfate per protein is expected in fraction V than in fraction II. The measured ratio, about 1.65*, is significantly

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* See footnote page 107.

higher. This discrepancy suggests that the recovery of serine in the amino acid hydrolysates of the two fractions may not represent the same percentage of the actual serine content in each. Perhaps the hydrolysis rate of serines which are substituted with polysaccharide chains is different from serines which are not substituted, or the destruction of serine during acid hydrolysis may be influenced by the relative amount of polysaccharide present. Such possibilities may account for the fact that the recovery of serine in fraction II was 10% lower than in fraction V (columns 1 and 2 of Table VII above) whereas none of the other amino acids showed as large a variation.

In sum, more data will be required before the results of the base elimination experiment can be satisfactorily interpreted. Tentatively, however, the results indicate that differences in both the numbers of chondroitin sulfate chains per molecule and in the average lengths of the chains contribute to the molecular weight polydispersity of PGS.

7. General discussion

Mathews and Lozaityte, 1958, proposed one of the first hydrodynamic models for bovine nasal proteinpolysaccharides. From light scattering data, they suggested that the subunit proteinpolysaccharide molecule had a root-mean-square radius of gyration, R_g, of about 1000 Angstroms and a molecular weight of 4 million. They made the assumption that the macromolecules contained no associated solvent, and then used this value of the molecular weight in combination with the value of the intrinsic viscosity for the sample to calculate an axial ratio of 90 for the solute species. From this axial ratio and the value for R_g, the authors suggested that the subunit was a rod about 3,700 Angstroms long. The shape of the $P(\theta)$ curves from their light scattering data was consistent with their model of rod-like particles. The experiments described in this chapter indicate that the assumption that macromolecules have no associated solvent in solution is incorrect. The molecules in the PGS fraction have physical characteristics which suggest that they are highly hydrated in solution and have axial ratios of less than 4; the

molecules are very polydisperse and have an average molecular weight of about 2.7 million. It is probable that the results presented by Mathews and Lozaityte were complicated because their preparations contained some aggregate and perhaps a small amount of collagen. One of their preparations was purified somewhat by removing larger particles with centrifugal techniques; this preparation would be expected to contain the least amount of aggregate. The molecular weight of this fraction was 1.7 million, and R_g was 830 Angstroms. If this preparation consisted of subunit macromolecules only, these values would indicate that subunits are somewhat asymmetric because a spherical model for PGS would predict a value of R_g no greater than 300-400 Angstroms for the physical data shown in Figure 23 above.

Mathews and Lozaityte also measured the molecular weight and R_g for a preparation which contained a large amount of aggregate; the values were 30 million and 2,400 Angstroms respectively. The authors postulated that aggregation of the rod-like subunits must occur laterally as well as end-to-end in order to account for this high molecular weight, but low R_g for aggregate. The physical data for PPC presented in this chapter were consistent with a model for the aggregate in which an average of about 13 spherical PGS molecules were arranged in a staggered array to form a prolate ellipsoid with an axial ratio of about 8. In solution such an aggregate complex would have an average molecular weight of around 35 million and an R_g of about 2500-3000 Angstroms. These values are fairly consistent with the results of Mathews and Lozaityte. However, light scattering measurements for PPC and PGS will have to be made to clear up some of the ambiguities between their data and those described in this chapter. The $P(\theta)$ function should be particularly interesting for PGS; if the macromolecules do indeed occupy spherical domains in solution this function should give a straight line when graphed against $\sin^2(\theta/2)$ even though the macromolecules are polydisperse.

Bernardi, 1957, concluded that a rod-like model for proteinpolysaccharides was unlikely because the macromolecules exhibited negligible

flow birefringence. He suggested that the physical data for protein-polysaccharides were consistent with a polydispersed system of unbranched coils. Such a model had been suggested previously by Webber and Bayley, 1956, who proposed that the structure of proteinpolysaccharides consisted of polysaccharide chains alternately linked in end-to-end arrangement with polypeptides. This model was based on the facts that the macromolecules exhibited some flow birefringence in water but not in salt solutions and that they were extensively depolymerized by treatment with trypsin. In the aqueous solution the molecules would be extended because of charge repulsion between the ionized groups within the macromolecules, while in salt solution, such charge effects would be minimized and the molecules would be more coiled. This model of unbranched alternating polysaccharide and protein is incorrect as is demonstrated by the inability of hyaluronidase to fragment the macromolecules in the same manner as trypsin (Mathews and Lozaityte, 1958).

The model for proteinpolysaccharides proposed by Luscombe and Phelps, 1967a, is very similar to that postulated for PGS in this chapter. The authors suggested that the macromolecules are highly solvated, occupy roughly spherical domains with an average radius of gyration of about 1,400 Angstroms, and have molecular weights between 3.2-5.8 million. The value of 3.2 million was determined from the intrinsic viscosity of their preparation and a value for the sedimentation coefficient for the sample which was determined by extrapolating peak s values observed at different solute concentrations to zero concentration. The value of 5.8 million was calculated from light scattering data. Because the value of the molecular weight estimated from light scattering is a weight average molecular weight, M_w, whereas that estimated from intrinsic viscosity and sedimentation coefficient is less than M_w, the authors suggested that the results indicated solute polydispersity. However, their preparation contained some aggregate complexes. This is indicated from the results of another experiment they describe in which the viscosity was about 80% less at pH 3 than it was at solvent pH values greater

than pH 5.5. Figure 12 above shows that the viscosity of PGS is almost constant from pH 2.3 to pH 8.4. The viscosity of PPC, on the other hand, was lower at pH 2.3 than at pH 5.8 because aggregate complexes dissociated at the lower pH value. The proteinpolysaccharide preparation of Luscombe and Phelps, then, must have contained 10-15% aggregate. This amount of aggregate could easily be overlooked in the ultracentrifuge, but it would have a large effect on the light scattering measurements, and lead to estimates of M_w and R_g which are too high. Therefore, the quantitative results these workers present are different from those described for PGS; however, the qualitative model for the hydrodynamic characteristics of the macromolecules is the same.

Further work is required to verify some of the details of the physical and chemical characteristics of PGS and PPC which were described in this chapter. Nevertheless, the data clearly indicate that PGS constitutes a polydisperse class of macromolecules with a broad range of molecular weights. The primary cause for the polydispersity was shown to be due to differences in the amount of chondroitin sulfate per protein content in the macromolecules.

CHAPTER V

A MODEL FOR PROTEOGLYCAN SUBUNIT

The macromolecules in the proteoglycan subunit fraction represent 80% of the proteinpolysaccharide content of bovine nasal cartilage. They interact with molecules in the glycoprotein link fraction and, as a result, form large aggregate complexes. Such complexes appear to be important for the structure and organization of the ground substance of the tissue. The PGS macromolecules are polydisperse and have high molecular weights, 1-4 million. They have complex chemical structures, in which chondroitin-4-sulfate, protein and keratan sulfate are all covalently bound into one type of molecular species. Figure 26 presents a schematic model for PGS and for the interactions between PGS and GPL which are responsible for aggregation. The purpose of this chapter is to summarize briefly the evidence for this model.

Hydrodynamic measurements in chapter IV indicate that an average PGS macromolecule has a molecular weight of 2.7 million. About 7% of its total weight is protein. If this protein is in the form of a single, long polypeptide as is drawn in Figure 26, it would have a molecular weight of 190,000. This is a large molecular weight for a protein, and it is quite possible that there are more than one polypeptide built into the structure of a subunit. About 50% of the amino acid residues in PGS are serine, glycine, proline and glutamic acid in approximately equal amounts. Anderson, Hoffman and Meyer, 1965, presented evidence that these four amino acids reside in the peptide regions where the chondroitin sulfate chains are bound to the protein core; the attachment was described as a glycosidic bond between the reducing end of a polysaccharide chain and the hydroxyl of a serine. A protein with a molecular weight of 190,000 and the amino acid composition indicated for PGS in Table IV would contain 200 residues of serine. The results of the base elimination experiment described in chapter IV suggest that an average of 45% of these serines, or about 90 residues, have chondroitin sulfate chains attached. The keratan sulfate chains which comprise 8% of the weight of PGS may also be attached directly to the protein, although the type of chemical bond involved is not known for certain. A large proportion of the protein moiety of PGS, then, serves a structural function of binding polysaccharide chains into the macromolecule.

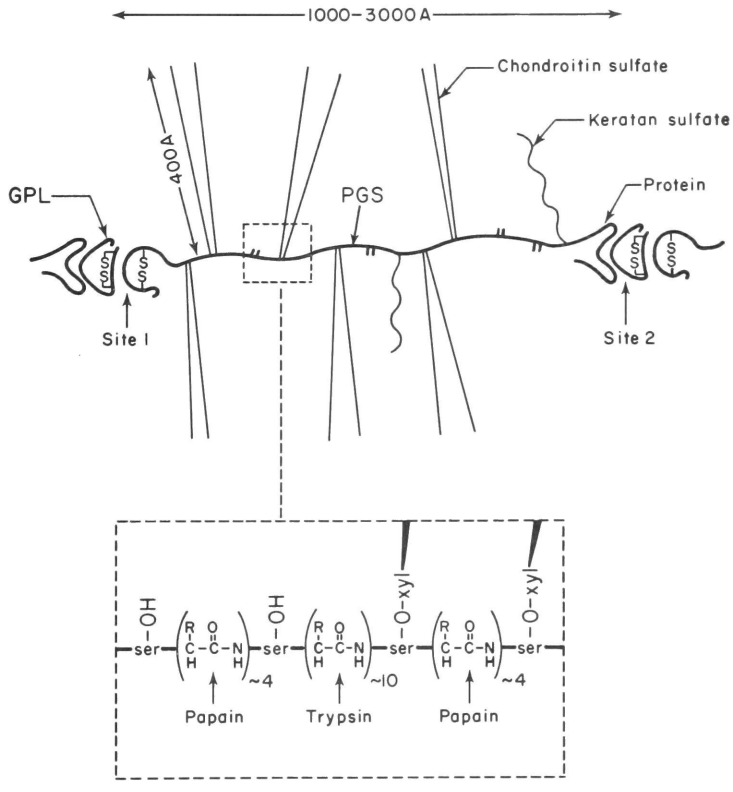

Figure 26. Schematic model of PGS and its interactions with GPL. See text for discussion.

The data presented in chapter III suggest that the interactions between PGS and GPL which result in aggregation are mediated through the conformations of the proteins in both components, and that at least two distinct interaction sites exist. The portions of the protein in PGS which interact with glycoprotein might reside at either end of the polypeptide as is drawn in Figure 26. In such a configuration, the chondroitin sulfate chains would radiate out from the middle portion of the protein, and this would leave the ends more accessible to the GPL molecules. One of the interactions between PGS and GPL, labelled 'site 1' in the figure, is abolished when aggregate is treated with sulfhydryl reducing agents; and the interaction is reversed by solvents with pH values below pH 4 or by solvents with high salt concentrations. The other interaction, labelled 'site 2', is unaffected by these treatments. Both interactions are reversed in the presence of 4 M guanidinium chloride. The results suggest that the protein conformations for PGS and GPL involved in interaction at site 1 require intact cystine disulfide bridges and that acidic groups, possibly glutamate or aspartate, are involved. The interaction at site 2 may involve hydrophobic regions within the proteins.

Eighty-five percent of the weight of PGS is chondroitin-4-sulfate, which exists as unbranched chains with the repeating disaccharide structure shown in Figure 2. If there are 90 chains in a subunit with a molecular weight of 2.7 million, the chains would have an average molecular weight of about 25,000. This value is in good agreement with reported values for the molecular weight of chondroitin sulfate prepared from nasal protein-polysaccharides. The extended length of a chain with a molecular weight of 25,000 would be 350-400 Angstroms. If the chains radiate out from the protein core, this length would be an estimate of the radius of the solvent domain the PGS macromolecule would be expected to occupy. This value is in good agreement with the hydrodynamic data presented in chapter IV, which indicate that the radius of an equivalent sphere for PGS would be 390 Angstroms. The physical measurements also indicate that the PGS macromolecules behave in solution as if they were particles with axial ratios of less than four. The long axis of the molecule, then, would be expected to be less than 3000 Angstroms.

Luscombe and Phelps, 1967b, and Mathews, 1968a, describe results which indicate that the chondroitin sulfate chains are constructed onto the protein as doublets, two chondroitin sulfate chains with a short peptide between them. They found that the molecular weight of chondroitin sulfate which was prepared by digesting proteinpolysaccharides with trypsin was about 50,000, whereas that for chondroitin sulfate which was prepared by digesting proteinpolysaccharide with papain was only 25,000. They postulated that the protein core of the macromolecules contained short peptide regions between adjacent chondroitin sulfate chains which were split by treatment with papain but not by treatment with trypsin; larger peptide regions between these doublet structures were susceptible to proteolysis with trypsin. These results, which are schematically indicated in Figure 26, suggest that the protein core of the PGS macromolecule contains a large number of repeating amino acid sequences within the polypeptide.

The PGS fraction has a large molecular weight polydispersity primarily because the total amount of chondroitin sulfate bound to the protein varies from molecule to molecule. The base elimination studies suggest that both the number of chains attached to the protein and the lengths of the individual chains in the macromolecule vary. The presence of the doublet structures just described indicates that the protein moiety of PGS must have pairs of serines within it which are available as chain initiating points. When the protein core is presented to the cellular apparatus which synthesizes polysaccharide, most likely located in the Golgi complex of the chondrocytes (Neutra and Leblond, 1966), some statistical average of the total number of serine pairs which can serve as chain initiators are used. The synthesis of a chain begins with a reaction between the serine hydroxyl and a uridinediphosphoxylose, UDP-xylose, to form xylosylserine; subsequently, activated sugars, UDP-galactose, UDP-glucuronic acid or UDP-N-acetylgalactosamine are added to this primer in the sequence outlined in Figure 3 (Robinson, Telser and Dorfman, 1966, and Grebner, Hall and Neufeld, 1966). Chain termination then occurs after an average of about 40 disaccharide repeat units are constructed on the growing chain.

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APPENDIX

Sedimentation coefficient distribution functions, g(s) and G(s)

In a centrifugal field, a boundary forms in a solution as the macromolecules sediment away from the air-solution meniscus (the macromolecules are assumed to be denser than the solvent). The velocity with which this boundary between solvent and solution moves in the centrifugal field provides valuable information about the hydrodynamic characteristics of the solute species. The velocity of the center of mass of the boundary divided by the centrifugal field, provides a measure of the sedimentation coefficient, s, of the solute. The value of s is characteristic of the molecules in the particular solvent and at the solute concentration of the experiment. However, the way in which the sedimenting boundary spreads as the experiment progresses can provide additional valuable information about the solute, and this is the basic information which the g(s) method is designed to exploit. Boundary spreading of solute in a centrifugal field can result from two factors: from diffusion of the solute during the time of the experiment and from solute polydispersity, *i.e.* from actual differences in the sedimentation characteristics of the molecules. When the contribution of diffusion to boundary spreading is negligible or when it can be corrected for, the g(s) technique provides an estimation of the range of sedimentation coefficients exhibited by the solute molecules under the particular conditions of the experiment. This information can be used to estimate the extent of molecular weight polydispersity if the hydrodynamic characteristics of the macromolecules are known or postulated and if the sedimentation properties of the macromolecules as a function of solute concentration can be determined. Such information is particularly useful for proteinpolysaccharides, which are very polydisperse and have large molecular weights. A brief outline of the methods used to calculate the distribution functions follows.

Rayleigh interference patterns were photographed at different times after an experiment was initiated. The patterns were scanned

with a comparator, and the fringe position, y, was measured as a function of the x coordinate on the photographic plates; the x values are in one to one correspondence with radial positions in the centrifuge cell. The interference fringes in the boundary often move out of the picture, and optical clarity and accuracy require the scanner to shift in the y direction from a fringe near the upper edge of the pattern to another closer to the center. Such fringe shifts must be added back to the curves when the interference patterns are reconstructed. In most experiments, interference patterns were selected from times in the experiment in which plateau regions existed in the Rayleigh pattern to the left of the slowest sedimenting component and to the right of the fastest sedimenting component. The former plateau provides a zero concentration reference value and the latter, when it is corrected for fringe shifts and radial dilution of solute due to the sectored shape of the cell, provides a total solute concentration reference value. The difference between the two is used to measure the total concentration, n_0, in fringe displacements for the solute. When it was not possible to find both plateau regions in the same photograph, the final g(s) distributions were constructed from two sets of measurements; an early time point provided the value for the total solute plateau and a late time point provided a value for the zero concentration plateau. The x,y values from the interference pattern were loaded into a programmed Control Data 160G computer, along with values for the x position of the air-solution meniscus, x positions for reference points on the plate, the angular velocity of the rotor and the elapsed time of the experiment. The data were processed in the following steps.

The program first reconstructed the Rayleigh interference patterns in terms of fringe displacement, n, as a function of radial position in the cell, r. This involved, first, adding back the y value of the spacing between adjacent fringes at x positions where fringe shifts were made and, second, converting the x values to radial positions by using the reference points on the plate and the known optical constants of the photographic system for the centrifuge. The n,r points were then

numerically smoothed by estimating new values of n_i at each r_i with second order least squares fits through from 2-5 (n,r) pairs on both sides of r_i.

$$n_i = a_0 + a_1 r_i + a_2 r_i^2 \qquad (1)$$

where the a_i are the calculated polynomial coefficients. A tracing of the reconstructed Rayleigh patterns for 3 time points in an experiment with PGS (0.20% in 0.5 M guanidinium chloride) is shown in the upper left of Figure 27. The decrease in the value of the plateau to the right of the boundary at the later times is the result of solute dilution due to sedimentation in the sectored shape of the centrifuge cell. The derivative, dn/dr, for each r_i was determined numerically from the smoothed interference patterns by calculating second order least squares fits to the data as before. In this case the derivative of the polynomial is used.

$$(dn/dr) = a_1 + 2a_2 r_i \qquad (2)$$

The derivative curves for the three time points are shown in the lower left of Figure 27. They correspond to the type of patterns which would be observed in the centrifuge if schlieren optics were used.

The derivative distributions, g(s), were then calculated from the value of dn/dr.

$$\begin{aligned} g(s) &= (1/n_0)(dn/ds) \\ &= (1/n_0)(dn/dr)(dr/ds) \end{aligned} \qquad (3)$$

The value of dr/ds can be determined from the definition of s.

$$r = r_m \, exp(\omega^2 st) \qquad (4)$$

$$dr/ds = \omega^2 tr \qquad (5)$$

where *exp* is the exponential function, ω is the angular velocity in radians per second, r_m is the radial position of the meniscus, and t is the total time in seconds that the angular velocity has been applied to the sample after a correction is made for the acceleration time at the beginning of the experiment.

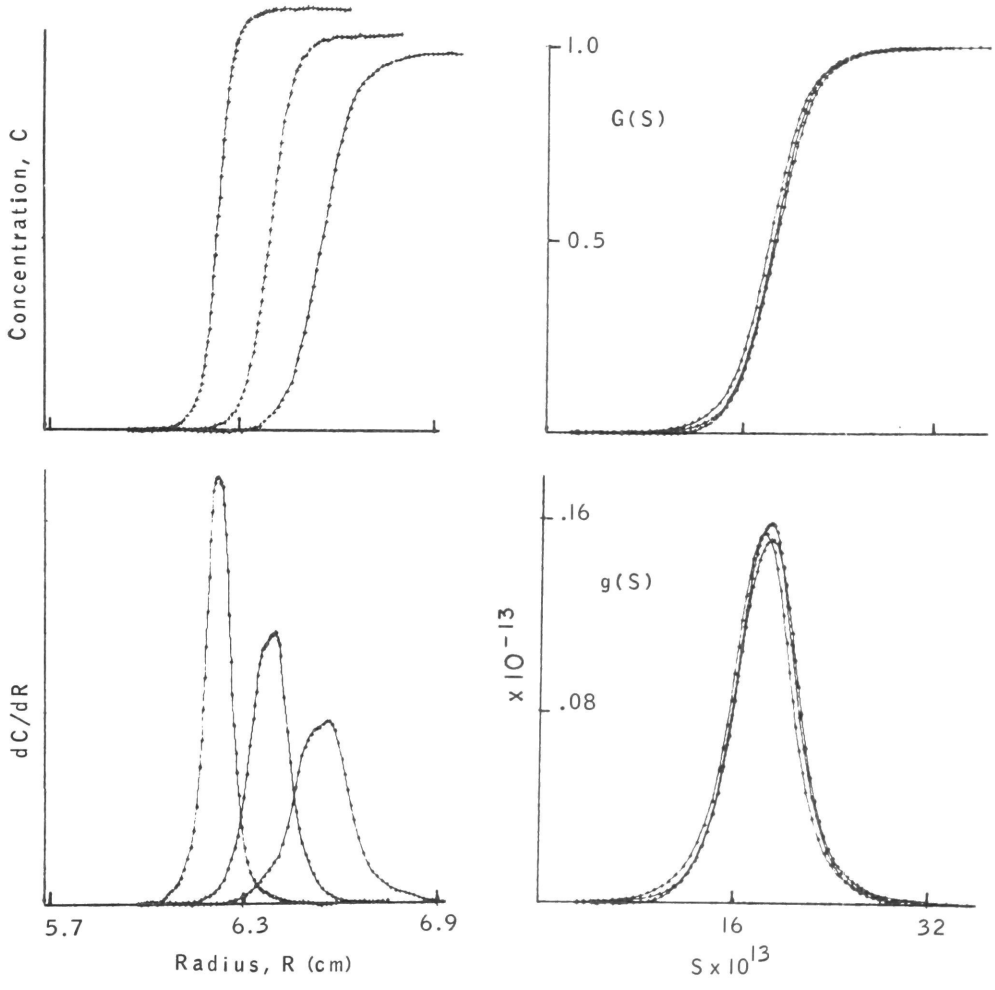

Figure 27. Different presentations of centrifugal data. The graphs are tracings of centrifugal data calculated by the procedures described in the text with a Control Data 160G computer. The solution was PGS (0.20%) in 0.5 M guanidinium chloride, 0.05 M MES, pH 5.8. The graphs in the upper left show the reconstructed interference patterns for three time points in the experiment. The graphs in the lower left show the derivative curves for the three times. The graphs in the lower right indicate the derivative, g(s), profiles and those in the upper right indicate the integral, G(s), profiles for the three times.

$$t = t_t - (2/3)t_i \qquad (6)$$

where t_t is the total elapsed time and t_i is the acceleration time required to reach the final rotor speed. A correction term, r^2/r_m^2, is introduced into the final equation for g(s) to correct for radial dilution.

$$g(s_i) = (1/n_0)(r_i^2/r_m^2)\omega^2 t r_i (dn/dr) \qquad (7)$$

where the value of dn/dr is taken from equation 2. The three patterns in the lower left of Figure 27 are now transformed into the three g(s) curves plotted in the lower right by calculating values of $g(s_i)$ from equation 7 and values of s_i from equation 4.

The integral distributions, G(s), were then calculated.

$$G(s_i) = (1/n_0) \int_0^{s_i} g(s)ds \qquad (8)$$

Equations 5 and 7 are substituted into equation 8.

$$G(s_i) = (1/n_0) \int_{r_m}^{r_i} (r^2/r_m^2)(dn/dr)dr$$

$$= (1/n_0) I(r_i) \qquad (9)$$

where I indicates the integral. The plateau region near the meniscus makes dn/dr = 0 at these radial positions, and I(r) = 0 for r near r_m. The integration in equation 9 is accomplished by a summation process across the boundary from solvent plateau to solute plateau. Each step in the summation involves calculating the integral between r_i and r_{i+1} and adding the value to $I(r_i)$.

$$I(r_{i+1}) = I(r_i) + \int_{r_i}^{r_{i+1}} (r^2/r_m^2)(dn/dr)dr \qquad (10)$$

The value for dn/dr from equation 2 is substituted into equation 10; the coefficients a_1 and a_2 are those which were calculated for dn/dr at r_i.

$$I(r_{i+1}) = I(r_i) + \int_{r_i}^{r_{i+1}} (r^2/r_m^2)(a_1 + 2a_2 r)dr$$

$$= I(r_i) + (1/r_m^2)[(a_1/3)(r_{i+1}^3 - r_i^3) + (a_2/2)(r_{i+1}^4 - r_i^4)] \qquad (11)$$

The plateau region to the right of the fastest sedimenting boundary again makes dn/dr = 0 for r greater than some r_p. This makes $I(r_p)$ a maximum. The G(s) distribution is normalized, which means that its maximum value is defined as 1.

$$G(s_p) = (1/n_0)I(r_p) = 1 \qquad (12)$$

Therefore n_0, the total fringe displacement, is equal to $I(r_p)$, and this value is actually the one used in calculating both the $g(s_i)$ and $G(s_i)$ values in equations 7 and 9 respectively. The three G(s) curves for the experiment are shown in the upper right of Figure 27.

The three g(s) and G(s) curves are from three different time points during the experiment; the sets of curves superimpose within experimental errors. If diffusion had contributed to the boundary spreading in any significant amount during the experiment, a profile calculated for an early time point would be broader than that calculated for any later time. This is due to the fact that sedimentation velocity of solute in the boundary is proportional to time whereas any diffusion of solute in the boundary is only proportional to the square root of time. This means that solute diffusion contributes relatively less to boundary spreading at later times than does solute sedimentation. In no centrifugal experiment with proteinpolysaccharides did g(s) profiles from early times in an experiment differ from those at later times more than would be expected from experimental errors. The contribution of diffusion to the profiles is therefore unimportant to the shapes of the curves, and the curves reflect real solute polydispersity in terms of solute sedimentation coefficients.

Concentration dependence of solute sedimentation velocities occurs for macromolecules when solutions with finite solute concentrations are centrifuged. Such concentration effects can be very large for hydrated,

high molecular weight polyelectrolytes, such as the proteinpolysaccharides. Hypersharpening of the boundary for PGS becomes quite pronounced at concentrations greater than 0.15% (Figure 19 above) and can lead to erroneous conclusions about the extent of polydispersity of the preparation. Thermodynamically meaningful sedimentation coefficients for a solute in a particular solvent can be made by measuring the s values at a number of loading concentrations and extrapolating to zero concentration. If this could be done for the sedimentation coefficients across the entire g(s) distribution for polydisperse macromolecules, an hypothetical ideal g(s) distribution would result in which concentration effects due to particle-particle interactions would be eliminated. The extrapolated profile would then reflect the actual range of sedimentation coefficients in the sample for the particular solvent. A procedure for extrapolating sets of g(s) data at different concentrations to zero concentration has been developed. It is based on a method suggested by J. L. Oncley (personal communication). The procedure uses the integral form of the distribution function, G(s).

A particular value of G(s) corresponds to the same weight fraction of solute for any total solute concentration; however, the sedimentation coefficients for that particular value of G(s) will be different for different solute concentrations. For example, G(s) values between 0.0 and 0.1 always represent those 10% of the macromolecules which sediment with the lowest sedimentation coefficients; but the values of the sedimentation coefficients for the macromolecules in this fraction will be less when the total solute concentration is greater because of the concentration effects on sedimentation velocities. For each G(s) value, a series of sedimentation coefficients, s_j, were calculated at a number of different loading concentrations, c_j. These values were extrapolated to zero concentration by fitting the (s_j,c_j) data to 2nd order least squares polynomials. When this is repeated for a number of values of G(s) between 0 and 1, the extrapolated ideal G(s) distribution is reconstructed. The results from such an extrapolation for PGS are described in Figure 23 above.

The precision of the extrapolation is uncertain at this time. It depends upon how well the (s_j, c_j) data are fit by second order polynomials and upon how precisely the sedimentation velocity profiles can be calculated for very low solute concentrations (down to 0.04%). Extrapolation of sedimentation coefficients against concentration is often done as a linear function of 1/s versus c (Williams, *et. al.*, 1958). Linear extrapolation of the data in the form of $(1/s_j, c_j)$ gave essentially the same results for PGS as those shown in Figure 23 above. The extrapolation has not been attempted for PPC because the proportion of the macromolecules that appear to sediment in the aggregate peak changes as a function of solute concentration (Johnston and Ogston, 1946), which makes the extrapolation procedure unreliable.

In sum, the g(s) method has been used throughout the thesis to evaluate semiquantitatively the differences between proteinpolysaccharide preparations which have been treated in a variety of ways. Care was taken in individual experiments to reproduce, when possible, the solute concentrations and solvent conditions in order that differences observed in the g(s) profiles can be attributed to real differences in the sedimentation properties of the macromolecules even under non-ideal conditions. In combination with rheological data, the method has provided a useful tool for estimating the extent of molecular weight polydispersity of the proteinpolysaccharide fraction, PGS.

End