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INSOLUBLE NON-COLLAGENOUS GLYCOPROTEINS FROM CARTILAGE

by

DONALD WINSTON SHIPP

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ABSTRACT

Two insoluble non-collagenous glycoprotein fractions (A and G) have been separated from puppy rib cartilage using mild selective techniques involving extraction of most of the proteoglycan and digestion of the insoluble residue with purified collagenase.

Both A and G form single bands in caesium chloride gradients but they differ in density. Both fractions are high in aspartate plus glutamate and have a low hydroxyproline content. Insoluble A and G contain hexose, glucosamine, sialic acid and chondroitin sulfate glycosaminoglycan, but differ in their contents of hexose and six amino acids. A non-covalent association exists between the insoluble glycoprotein fractions and some of the chondroitin sulfate glycosaminoglycan.

The solubility properties and amino acid compositions of Fractions A and G are similar to those of structural glycoproteins (SGP) extracted from other tissues.

After reduction, alkylation and extraction with SDS most of each protein is solubilized. Gel electrophoresis of solubilized A or G shows the presence of either one or two bands and gel chromatography shows both high and low molecular weight peaks. The production of a low molecular weight electrophoresis band from the high molecular weight Sephadex fraction indicates that there is aggregation and disaggregation of subunits in SDS. Aggregate formation could not be demonstrated by gel chromatography or analytical ultracentrifugation alone. Using the latter technique, SDS solubilized A and G sediment as single symmetrical peaks but with different S values.

Biochemical and electron microscopic evidence shows that both insoluble fractions are in close association with insoluble collagen. Electron microscopy shows that both insoluble glycoprotein fractions stain with lead, ruthenium red or alcian blue plus phosphotungstate and that Fraction G contains many fine filaments whereas Fraction A appears amorphous. Material with the same appearance and staining properties was found to occur on the surface of collagen fibers in the undigested cartilage residue.

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LIST OF ABBREVIATIONS

g	: acceleration due to gravity
Acid GAGs	: acid glycosaminoglycans
Alcian	: alcian blue
A°	: Angstrom units
bis	: N, N'-methylenebisacrylamide
β-ME	: beta mercaptoethanol
BSA	: bovine serum albumin
^{14}C	: carbon 14
cm	: centimeter
p	: confidence limits of the student 'T' test
cpm	: counts per minute
°	: degrees celsius
Disc	: discontinuous
dpm	: disintegrations per minute
DTT	: dithiothreitol
EDTA	: ethylenediaminetetraacetic acid
Fr. 1	: Fraction 1
g	: gram
h	: hour
GPL	: link glycoprotein
µ	: micron
µg	: microgram
µl	: microliter
m	: milli
ma	: milliamperes
mCi	: milliCurie
mg	: milligram
ml	: milliliter
mM	: millimolar
min	: minute
M	: molar
M.W.	: molecular weight
NCPA	: non-collagenase proteolytic acitivity

OD_{500}	: optical density at 500 mμ
PTA	: phosphotungstic acid
PPC	: protein polysaccharide complex
PGS	: proteoglycan subunit
r_{av}	: radius (average)
RPM	: revolutions per minute
sec	: second
SDS	: sodium dodecylsulfate
SGP	: structural glycoprotein
S.E.	: standard error
S	: Svedberg unit
TEMED	: tetramethylethylenediamine
TCA	: trichloroacetic acid
U	: unit

CHAPTER I LITERATURE SURVEY

1. General Introduction

Connective tissues are composed of cells separated by extracellular * matrix. The types of cells and extracellular matrix produced by them varies in different connective tissues and reflects their particular function. The extracellular matrix consists of fibrous proteins (elastin, collagen) and a non-fibrous component or ground substance. Ground substance is composed of proteoglycans of the acid glycosaminoglycans (5), glycoproteins (1, 11) and interstitial fluid containing inorganic components (9). Interactions between the components of the ground substance at the biochemical (1, 3, 5, 12, 13) and ultrastructural (75) level as well as interactions between ground substance and fibrous components (85, 90, 91, 96, 103, 110, 131, 154) have been the subject of many studies. Total understanding of these complex interactions awaits the complete chemical elucidation of all the extracellular matrix components, their biosynthesis, pathways of secretion and degradation. How all of these factors change during development is critical in order to understand the integrity of the connective tissue at any point in time.

A knowledge of the macromolecular organization of cartilage extracellular matrix is essential to understand its normal functional properties which lie in its resilient resistance to deformation and compression. Degradation of ground substance components in articular cartilage occurs in osteoarthritis and rheumatoid arthritis and degradation of cartilage matrix has been the objective in treating disc disorders (190). A complete view of the pathogenesis of these diseases must involve a description of the enzymes involved in the degradatory process and the events leading to the synthesis

* The term intercellular also is used in many texts.

and release of these enzymes from the chondrocytes. Such studies require the isolation of well characterized substrates from the extracellular matrix in order to investigate these diseases.

2. Fibrous Components

Collagen is the main fibrous component of cartilage extracellular matrix. The primary structure and biosynthesis of collagens from several connective tissues have recently been reviewed (33, 34). The cartilage collagens are of the type $[\alpha 1(II)]_3$ as opposed to the more common collagen composition $[\alpha 1(I)]_2\alpha 2$ type. The $\alpha 1(II)$ chains differ from $\alpha 1(I)$ chain in certain amino acids and carbohydrate composition (35).

3. Ground Substance

(a) Acid Glycosaminoglycans (Acid GAGs)

The chemistry and distribution of acid GAGs has been well reviewed (3, 5, 36, 46). Table I shows the chemical composition of the disaccharide repeating units of the acid glycosaminoglycans. Early studies on the composition of bovine nasal cartilage indicated "the major portion of the cartilage is a protein salt of chondroitin sulfuric acid" (37). Since then the presence of dermatan sulfate, chondroitin-4 and 6-sulfate has been demonstrated in many other connective tissues (36, 38, 47).

Keratan sulfate was first isolated from cornea (39), however, it is present in numerous other tissues including mammalian cartilage (40, 41, 50) where it appears to be part of the chondroitin-sulfate-protein complex (41, 42). In human costal cartilage (43), bovine nasal cartilage (45) the keratan sulfate content increases from zero at birth to high levels with old age.

At the same time there is a drop in the chondroitin sulfate content. Mathews and Glagov (44) also have examined the composition of human cartilage with aging.

Hyaluronic acid is found in a number of connective tissues and interstitial fluids (particularly skin and synovial fluid). In cartilage it is a minor component (13, 48, 49, 153) and it is involved in proteoglycan aggregation (77).

Heparan sulfate has not been found in cartilage, however, it has been extracted from bovine (51) and human (52) aorta where it has been shown to occur as a proteoglycan consisting of several polysaccharide chains of heparan sulfate linked to a central protein core.

(b) Proteoglycans

Using the nomenclature of Hascall and Sajdera (12), proteoglycans are macromolecules which consist primarily of polysaccharides covalently bound to a small amount of protein, whereas glycoprotein refers to macromolecules which are primarily protein with covalently bound saccharide. For many years it had been recognized that chondroitin sulfate does not exist as free polysaccharide in cartilage (53). Shatton and Schubert (54) were the first to prepare a chondroitin sulfate non-collagenous protein complex (proteoglycan) from bovine nasal cartilage. They suggested the chondroitin sulfate and protein are covalently linked. Since this time proteoglycans have been isolated by many investigators using a variety of techniques (11, 55, 56, 57, 58, 59, 60). Proteoglycans have been isolated containing chondroitin-4 and 6-sulfate (54, 73), dermatan sulfate (38), keratan sulfate (41) and heparin (51, 52).

Cartilage ground substance is composed mainly of proteoglycans containing chondroitin-4-sulfate, keratan sulfate and chondroitin-6-sulfate. Hyaluronate and a number of glycoproteins also have been found.. Caution should be exercised when deciding whether isolated fractions from the ground substance are 'glycoprotein' as distinct from proteoglycans containing keratan sulfate. Studies on the keratan sulfate prepared from bovine nasal cartilage subunit (32) show that it contains neutral sugars (galactose, manose, fucose), sialic acid, hexoseamines (glucosamine, galactosamine). However, the galactose and glucosamine are in approximately 1:1 ratio as in the repeating disaccharide of keratan sulfate.

Structural studies on the chondroitin sulfate proteoglycan macromolecule indicates that it consists of a polypeptide backbone with glycan side chains radiating from the protein core (61, 62, 63). The chondroitin sulfate chain may consist of a mixture of disaccharide repeating periods, each one with either no sulfate or sulfate at the C-4 position of n-acetyl galactosamine (N-Ac gal) (64, 65). In addition, keratan sulfate type of glycan (66, 67, 76) seems to be attached to the same protein core which carries the chondroitin sulfate, however, some chondroitin sulfate proteoglycans do not contain keratan sulfate (70). In rabbit and human costal cartilages these glycan components vary with age. In that the functional roles of the different glycan chains is unknown, the biological significance of these changes is not known.

The chondroitin sulfate chains are covalently attached at their reducing ends to the protein core via a well defined glycosidic linkage: (galactose-galactose-xylose) to the hydroxy group of serine (59, 71, 72) or

threonine (73). Keratan sulfate is attached to the protein core with a glycosidic bond between galactosamine and serine or threonine (32) as well as an alkali stable bond thought to involve glutamic acid.

In bovine nasal cartilage the basic structural unit is the proteoglycan subunit (PGS) (12). It is considered that this subunit is polydisperse as a result of variations in the length and numbers of chondroitin sulfate and keratan sulfate chains bound to the protein core. The molecular weight ranges from 2.5×10^5 to 4×10^6 (74). The protein core has a molecular weight of about 200,000 with an average length of 3,400A^{o} (75).

The present understanding of the macromolecular organization of proteoglycans comes primarily from studies on bovine nasal and pig laryngeal cartilage. It is thought that proteoglycans exist in vivo in the form of high molecular weight aggregates consisting of a non-covalent association of at least three biochemically defined species; proteoglycan subunit, link glycoprotein (GPL) (12) and hyaluronic acid (77, 78, 79, 80, 81). It is noteworthy that the identification of hyaluronic acid in such aggregates is the first indicator that free glycosaminoglycans may play a role in the organization of ground substance. The large molecular weight aggregate can be dissociated into its smaller molecular weight components by dissociative solvents (4M guanidine HCl) and upon recombination of the components in associative solvents (0.5M guanidine HCl) the aggregates have been visualized in the electronmicroscope (75). Studies with bovine articular cartilage (1) indicate some differences in the macromolecular organization. In this tissue a higher level of proteoglycan organization has been shown to exist involving proteoglycan aggregate (similar to that in bovine nasal cartilage), collagen and an uncharacterized 2.7S protein.

(c) Collagen-Proteoglycan Interactions

A good deal of evidence now exists in the literature which indicates that ground substance components interact with fibrillar collagen in the formation of a functional matrix. In vitro experimentation has indicated non-covalent associations of ground substance with collagen, but in some cases where a very tenacious association has been demonstrated between non-collagenous material and collagen in tissue homogenates and digests there is evidence for a covalent attachment.

(1) Biochemical Studies

(i) Nature of the Association

In the sea anemone Metridium dianthus a covalent linkage between a heteropolysaccharide and a hydroxyproline containing peptide has been isolated (82) from a collagenase digest of pepsin solubilized collagen, thus providing direct evidence for a covalent linkage in this tissue. Covalent linkages have been suggested between shark collagen and proteoglycan (83, 84); between collagen and keratan sulfate and between collagen and proteoglycan core protein in human costal cartilage (85). In these cases the covalent bond has not been isolated. Good evidence (110) is present for an ionic interaction between collagen and proteoglycan in human intervertebral disc. Different tissues and techniques were used by Kobayashi and Pedrini (85) and Steven et al (110) to prepare the proteoglycan-collagen fractions. The contrasting results could be due to tissue differences or alternatively the preparatory technique. For example, recent data from bovine articular cartilage (111) indicates that destruction

of collagen helical structure occurs upon extraction with guanidine HCl when the 'optimum' concentration of the salt for proteoglycan extraction is exceeded. This creates undefined new interactions between the denatured collagen and proteoglycan which is reflected in the irreversible insolubilization of the latter. Since Kobayashi and Pedrini (85) used this optimum concentration (4.0M guanidine HCl) to extract proteoglycan, the implicated covalent association between collagen and unextractable proteoglycan may be artifactual. That some denaturation of collagen occurs in 4.0M guanidine HCl is consistent with the observations of Anderson and Sajdera (92). Studies by Steven et al (110) were with proteoglycan prepared in a different manner. As well the investigators may have been looking at different soluble proteoglycan pools. Further support for an ionic interaction between collagen and acid GAGs of proteoglycan is documented (103, 112, 113).

(ii) Effect on Collagen Fibril Formation

Early biochemical studies show an association of collagen with acid GAGs (131) and with proteoglycan (16, 102, 104). Tropocollagen molecules can aggregate under physiological conditions in vitro to form gels containing mature collagen fibers (100). Collagen fiber formation in vivo takes place in the extracellular matrix (101). Studies have shown that dermatan sulfate proteoglycan (38) or chondroitin sulfate porteoglycan (103, 105) modify the kinetics of collagen fibril formation. Aggregated proteoglycan (PGS and GPL) does not (105). On the basis of these kinds of experiments it was suggested that the physical state of proteoglycans is important in controlling the initial stages of collagen fibril formation. It also has been hypothesized that proteoglycans play a role in collagen fibril stabilization (106, 107, 108)

and may be an important factor in the organization and function of connective tissue at all stages of growth and development.

On the other hand, it has been shown that although the body wall of the sea anemone Metridium dianthus (109) contains no proteoglycans, yet the collagen fibers present are similar, with respect to several criteria, to those present in tissues which contain proteoglycan. This suggests that proteoglycans play no part in collagen fibril formation or stabilization in this organism, and possibly in others.

(iii) Metabolism

Gross, Mathews, Dorfman (197) and Bowness (198, 199) found that 70-80% of the chondroitin sulfate proteoglycan in rib cartilage can be extracted with water (198). The water insoluble chondroitin sulfate was removed from the residual insoluble collagen by alkali extraction. Using in vivo labelling techniques, the water soluble proteoglycan pool from rib cartilage (197, 198, 199) showed a higher specific radioactivity than that extractable with alkali. Using in vitro labelling techniques with pig laryngeal cartilage (192), calf rib cartilage (151, 196) the most highly labelled proteoglycans are those which were in firm association with collagen and hence unextractable with water or dissociative solvents. The reason for this difference in metabolic activity of the insoluble proteoglycan is not known, however, it does indicate metabolic heterogeneity of the total tissue chondroitin sulfate proteoglycan. This has been documented by others (193, 194, 195). With respect to the extractable proteoglycan pool, several studies (151, 196, 200) show that the chondroitin-4-sulfate chains of high molecular weight appear to be preferentially

synthesized and turnover independently of the lower molecular weight species.

(iv) Calcium Binding

It has been suggested by Boyd and Neuman (206) that the ion exchange properties of cartilage may be responsible for producing the local concentrations of calcium which might lead to the formation of hydroxyapatite crystals. Calcium binding by free chondroitin sulfate (201, 202, 205, 207) and chondroitin sulfate proteoglycan (202, 204, 208) has been documented. Dunstone (205, 207) found that the order of increasing cation affinity for both chondroitin-4-sulfate and cartilage to be $K^{+} < Na^{+} < Mg^{++} < Ca^{++}$. It seems likely that immobilization of Ca^{++} is, in part at least, the result of site binding as opposed to pure electrostatic attraction (202, 208). Studies with chondroitin sulfate (203) and chondroitin sulfate proteoglycan (214) have shown that these macromolecules can react with calcium phosphate and prevent it from precipitating from solution. Sobel et al (209, 211) have shown that chondroitin sulfate in association with collagen can act as a nucleating centre for the growth of hydroxyapatite crystals. Bowness (215) found that the water insoluble chondroitin sulfate had the greatest affinity for calcium. This pool decreased the amounts of calcium and phosphate precipitated from solution, however, the formation of calcium phosphate from calcium bound to this pool proceeded more rapidly than from calcium in solution. In rib cartilage, more of the total tissue chondroitin sulfate is found in the alkali soluble pool as calcification progresses (152).

To date no conclusive evidence exists to suggest any one calcification mechanism, nor should it be expected that any one mechanism is

operative at all times. Although a role for chondroitin sulfate in calcification has been suggested (209, 210, 211, 212) the proposed mechanisms are different; one suggesting initiation of calcification, the other suggesting inhibition. Whether the bound calcium and phosphate can react together and play a part in calcification or whether they are so tightly bound that they cannot react, thus producing inhibition of calcification, is an open question. Bowness (150) has proposed a model for the role of chondroitin sulfate glycosaminoglycan in calcification which incorporates the evidence for the nucleation and inhibition mechanisms.

(2) Electron Microscopic Studies

Electron microscopic observations on the ultrastructure of cartilage extracellular matrix indicate that there is an association of proteoglycan with collagen. Early studies by Martin (86) and Fitton-Jackson indicated the presence of fine fibrils (100-400A° diameter) which do not show the 640A° banding pattern characteristic of collagen. On the basis of their different morphology it was concluded that these fibrils were of a different chemical composition to collagen. In bovine articular cartilage (88), the distribution of proteoglycan and collagen indicated that the proteoglycan exists in the intercellular matrix in two states. Free and randomly oriented proteoglycans were predominant in the pericellular zone whereas proteoglycan attached transversely over the a and b bands of each 640A° period on the collagen fibers was observed in the main matrix. Smith (89) extended these observations to the epiphyseal plate cartilage in rabbit where the collagen fibers are of much smaller diameter (80-200A°)

than in bovine articular cartilage (500-600A°). He observed bismuth staining moieties (which in previous studies were identified as proteoglycan) (88, 90) which were tangentially attached to each period of the small collagen fibers of the matrix. Association of proteoglycan with collagen has been supported in guinea pig epiphyseal cartilage by Thyberg et al (91) using ruthenium red and alcian blue to show matrix granules in the intercellular matrix. These granules (100-800A° diameter) have been observed by others (91, 92) and in combination with biochemical techniques these granules were concluded to be ultrastructural representations of proteoglycans. Most granules are tangentially attached to collagen fibers, however, some were interconnected by poorly stained filaments which often projected from them. Associated proteoglycan-collagen has been observed in human synovium (94), aorta, articular cartilage, cornea (95) and bovine epiphyseal cartilage (96). In synovium four different types of ruthenium red positive structures are observed; amorphous coats, transverse belts, fine lateral filaments and intermediate fibrils. Fine lateral filaments were also observed in aorta and articular cartilage but not in cornea. Biochemical procedures to remove proteoglycans removed most of the associated ruthenium red material but left short lateral filaments attached to the collagen fibers. Eisenstein et al (96) examined the distribution of proteoglycan associated with collagen in calf scapular epiphyseal cartilage. They found at least two anatomical pools of proteoglycans; one which resides between the collagen fibrils and is extractable with dissociative solvents (4M guanidine HCl) and the other appear strongly attached to collagen which is not extractable by guanidine HCl. In conjunction with specific enzyme digestion procedures, the identity of ruthenium red material attached to

collagen could not be conclusively defined except that some was proteoglycan on the basis of its removal by testicular hyaluronidase or trypsin. The authors do not rule out the possibility that the ruthenium red particles associated with collagen is another anionic material left behind after extraction and/or enzymatic digestion.

(d) Non-Collagenous Proteins

(1) Occurence

Glycoproteins distinct from collagen and proteoglycans have been demonstrated in the extracellular matrix of many connective tissues such as bovine nasal septa (1, 11), corneal stroma (14), laryngeal cartilage (4), calf skin, calf joint, rabbit skin (15), bovine achilles tendon (17), human aorta (19), bovine ligamentum nuchae (146), bone (23, 114). Most of these glycoproteins or non-collagenous glycoproteins have been partially characterized biochemically, but, their functional role in the ground substance is not yet clear (12, 20, 114, 147).

In part the characterization of non-collagenous glycoproteins has been hindered by their complex interactions with other connective tissue macromolecules (collagen, elastin, proteoglycans) and perhaps other unknown interactions with macromolecules yet to be identified. The presence of non-collagenous glycoprotein in connective tissues was indicated by Bowes (25) where it was noted that the difference in composition between crude and purified collagen can be accounted for by non-collagen protein. Purification of a glycoprotein from human thoracic aorta was achieved by Barnes and Partridge (19) and they reported it had a high content of acidic amino acids. This feature appears to be common to all non-collagenous

connective tissue glycoproteins and affords a means of distinguishing them from plasma glycoproteins. In this chapter, an attempt has been made to categorize the known glycoproteins of connective tissue on the basis of their solubility properties.

(2) Soluble Matrix Glycoproteins

Some of the glycoproteins in the ground substance can be extracted with relatively mild procedures such as water, (21, 22); neutral or slightly alkaline solutions (24, 25, 26); dissociative solvents used for proteoglycan preparation (12, 26) or a combination of the latter two (17). Plasma glycoproteins have been shown to be present in the ground substance and are isolated by mild extraction procedures (28, 29, 30, 31, 114). It has been suggested by Ashton et al (114) that they may play a role in regulation of extracellular and membrane bound enzyme systems thus influencing cell metabolism and mineralization in bone.

Two glycoprotein fractions were isolated from bovine nasal cartilage by Hascall & Sajdera (12) during the course of investigation of proteoglycans in this tissue. These glycoproteins are isolated by relatively mild techniques which precludes cleavage of chemical bonds. Equilibrium density gradient fractionation of the cartilage extract under 'associative conditions' effects the isolation of 'glycoprotein - I' from the upper part of the gradient and aggregated proteoglycan (PPC) from the bottom. Glycoprotein - I has some collagen and uronic acid associated with it, however, the nature of these associations are not defined. The amino acid composition indicates a predominance of acidic over basic amino acids and that it

contains cysteine. This glycrprotein has not been studied in the same detail as the link glycoprotein (GPL) isolated from the same tissue. GPL participates in the aggregation of proteoglycan subunits (PGS). Disaggregation and isolation of PGS and GPL from PPC is effected by caesium chloride density gradient in the presence of dissociative solvent (4.0M guanidine HCl). GPL has a high content of acidic amino acids, contains cysteine, uronic acid but no detectable collagen. It shows two bands on SDS gel electrophoresis (116). Recombination of PGS, GPL and hyaluronic acid (13, 77, 117)reforms an aggregate under associative conditions. Treatment of GPL with dithiothreitol abolishes aggregate formation but does not prevent binding to PGS. GPL (in bovine tracheal cartilage) was further fractionated (118). GPL prepared from resting and ossifying regions of calf scapula cartilage show a single band on disc electrophoresis (119). Calcium binding studies show that it has two calcium binding sites and it is suggested that it may have a function in calcification (119).

Two glycoprotein fractions (A and B) distinct from serum glycoproteins, have been isolated from bovine achilles tendon by Anderson & Jackson (17) by exhaustive salt extraction. Most of the proteoglycan was removed from glycoproteins A, B by caesium chloride density gradient centrifugation leaving residual amounts associated with each. Glycoprotein A contained a trace amount of collagen, glycoprotein B considerably more. Both glycoproteins contained high contents of acidic amino acids and cysteine but differed with respect to several amino acids. They appeared heterogeneous on isoelectric focusing, but, in combination with tropocollagen showed a single band. As noted by the authors, the findings indicate that the

glycoproteins may play some part in stabilizing the tissue, perhaps by maintaining the structural stability of the collagen fibers above a certain diameter (120). Fractionation of glycoprotein B on affinity columns yielded components which can be dissociated into a proteoglycan fraction and a protein fraction by caesium chloride density gradient centrifugation in 4.0M guanidine HCl, indicating similar interactions to those which occur between GPL and PGS from bovine nasal cartilage.

Glycoproteins have been isolated from EDTA extracts of adult bovine cortical bone by Herring and others (27, 114, 127). Bone sialoprotein has an unusually high content of aspartate, glutamate and sialic acid. Its role is unknown although it has been shown to bind calcium, yttrium, thorium (122), plutonium and americium (123). Other glycoproteins have been isolated from bone EDTA extracts. Two fractions have been isolated which contain chondroitin sulfate bound by alkali labile linkages to a protein very similar in amino acid composition to bone sialoprotein. This data indicates a xylose-serine linkage of chondroitin sulfate to a protein core which is very different from that found in cartilage.

Polyacrylamide gel electrophoresis in combination with immuno-electrophoretic techniques have shown that at least four plasma glycoproteins, serum albumin, G-2-B glycoprotein, IgG and transferrin are present in EDTA extracts of bovine cortical bone (114). The G-2-B glycoprotein was shown to be immuologically identical to α1 globulin, however, the ratio of G-2-B glycoprotein to albumin was 250 fold higher in the bone collagenase digest (before extraction with EDTA) than in plasma; thus this glycoprotein appeared to be concentrated in bone tissue. The authors speculate that the G-2-B glycoprotein (on the basis of the similarity to the α1 globulins in plasma)

may have a role in regulating extracellular and membrane bound enzyme systems in the bone tissue.

Glycoproteins with covalently bound phosphate (phosphoproteins) have been isolated by Veis and others, from bovine dentin (124, 125, 126, 127), rat incisor dentin (128) and bovine enamel (129). In rat incisors, a relatively homogeneous phosphoprotein is readily solubilized from the insoluble collagen and has an unusually high content of aspartic acid and phosphoserine (over one-half of the total amino acid composition). In bovine dentin evidence was obtained for a covalent attachment of collagen to an insoluble phosphoprotein fraction. A second phosphoprotein fraction from this tissue is soluble in EDTA at neutral pH. The EDTA soluble fraction contains a number of proteins, one of which has a high aspartic acid and serine content similar to that from rat incisors but has considerably less covalently bound phosphate. This protein contains a covalently bound nucleotide like substance, however, its function is unknown (132). Data from bovine dentin suggests that there are several EDTA soluble glycoproteins as is the case in bovine cortical bone. In bovine dentin one component of the EDTA soluble portion is similar to the EDTA insoluble fraction which is covalently bound to collagen. Although calcium binding studies have not been done with these phosphoproteins their composition suggests their ability to bind calcium. In view of the ion binding properties of bone sialoprotein (133) it is possible that the phosphoproteins bound to collagen may have a role locating the deposition of mineral in the collagen matrix. In the soluble form they could bind and transport calcium (125) from the odontoblastic process through the predentin to the insoluble matrix. Electron microscopic evidence for this migration and for the localization of mineral

deposition is presented later in this survey. Studies by Carmichael (126) on phosphoprotein from bovine dentin confirms that presence of soluble and insoluble phosphoprotein fractions. The observation that xylose (not a usual sugar in glycoproteins) is present in the soluble phosphoprotein fraction in conjunction with a high galactose content suggest that part of the xylose and galactose may be derived from the linkage region of chondroitin sulfate proteoglycans.

(3) Insoluble Matrix Glycoproteins

(i) Isolation and Characterization

In a number of connective tissues there are glycoproteins which require more extreme conditions such as urea or solvents of high pH for solubilization (25, 135, 155, 156). Many of these glycoproteins are closely associated with collagen and have been solubilized only after prior removal of the collagen with hot TCA (14, 15, 136, 137, 138, 139). These solubilized glycoproteins were called structural glycoproteins (SGP) by Robert (138) or acidic structural proteins by Timpl (15). The isolation procedure involves removal of the soluble components with various salt solutions and/or buffers, treatment of the insoluble residue with hot TCA to remove insoluble collagen and then extraction of the insoluble residue with 8M urea. The urea soluble proteins from a variety of connective tissues exhibit similar amino acid compositions (137, 139), with high contents of acidic amino acids. Based upon the carbohydrate composition (142) metabolic behaviour, immunochemical properties (136) and amino acid composition, Robert (137) has proposed that this fraction represents a new class of connective tissue structural

proteins. The polysaccharide chains of the urea soluble SGP appear to be highly branched with a core containing di and trimannoside units together with N-acetyl-glucosamine (138).

The extraction procedure of Robert et al (137) was modified by Timpl et al (139). After removal of the urea soluble proteins the residue was extracted with 0.2N NaOH, which solubilized 70-98% of the non-collagenous structural proteins in the residue. The ratio of urea soluble to alkali soluble varied greatly from tissue to tissue. In rabbit skin it appeared that the alkali soluble proteins accumulate with aging. Very low levels of uronic acid were detected in both fractions suggesting that minute amounts of acidic GAGs were present. There is a close similarity in amino acid composition between the urea soluble and alkali soluble proteins with the exception that the alkali soluble fraction contains much less cysteine. That collagen removal is necessary before solubilization is indicated from the fact that significantly less acidic protein could be extracted from the various connective tissues before TCA treatment. As well the solubilized fraction has a high content of hydroxyproline (15). In that dissolution of collagen by hot TCA treatment may have some effect on the residual proteins (15), a less severe method was devised by Furthmayer (140) and Moczar (141) using collagenase digestion to remove collagen, followed by urea extraction. A urea soluble fraction was obtained and the residue extracted with a number of solvents (0.2N NaOH, $NaBH_4$ in 8M urea, βME in 8M urea). Although the extent of solubilization of βME - 8M urea was slightly less efficient in terms of protein extracted, it was considered the most desirable as it gave a preparation with solubility characteristics which were most similar to the proteins originally present in the connective

tissue (144). Since reduction of disulfide bonds effected solubilization it was suggested by Timpl et al (140) that disulfide crosslinks are in part responsible for the insolubility of these proteins. Thiol reagent alone was an ineffective solubilizer, indicating that disruption of the protein conformation also is necessary for solubilization. Both urea and urea-βME soluble fractions exhibited electrophoretic heterogeneity (up to 15 components). Molecular sieve chromatography of the urea soluble fraction also indicated heterogeneity. Immunochemical studies showed an antigen common to the urea soluble and the disulfide cross linked proteins in rat skin. This suggested a precursor role of some components in the urea soluble proteins which during development may become insolubilized by disulfide crosslinking (144). An acidic structural protein has been prepared from Metridium dianthus connective tissue (145) and accounts for at least 16.5% of the dry weight of the tissue. The amino acid composition of this partially purified preparation is similar to that reported for acidic structural proteins from mammalian tissues, however, it differs in that it is solubilized by 1-2% acetic acid.

A structural glycoprotein with slightly different solubility properties has been purified from experimental granulomas by Rajamaki and Kulonen (2). As with preparations described by Wolff et al (144) and Robert et al (137) the collagen was removed before solubilization could be effected, however, the glycoprotein was isolated from the material solubilized by the collagenase digestion. The insoluble residue was not examined. Separation by molecular sieving and ion exchange chromatography isolated a preparation free from collagen with an amino acid composition resembling that reported by Timpl et al (139).

Studies on elastin containing tissues have shown an acidic structural protein fraction can be isolated from thoracic aorta (141), bovine ligumentum nuchae (20, 146). In thoracic aorta the preparatory scheme was similar to that used by Robert and Compte (137) except that 0.1M βME was added to the 8M urea extracting solvent. The urea-βME soluble portion was fractionated into a water soluble and insoluble fraction. In general, chemical analyses of the two fractions were similar as well as their sedimentation velocity and electrophoretic mobility on Cellogel. In bovine ligamentum nuchae Ross and Bornstein (20) found that some acidic proteins were solubilized by treatment with 5M guanidine HCl in DTT. There were slight differences in composition of the extracted materials but the range of composition was similar to that found for previously isolated structural glycoproteins. In conjunction with electron microscopic studies, these solubilized proteins were identified (20) as microfibrils of approximately 110A° diameter in close association with a centrally located amorphous elastin component. Electron microscopy of bovine ligamentum nuchae showed removal of microfibrils with urea-βME (146), guanidine HCl-βME (20). The microfibrils are observable in mature elastic fibers from fetal and newborn calf ligumentum nuchae and this technique allowed their separation from elastic tissue. The amino acid composition of these microfibrils is similar to that of acidic structural proteins isolated from bovine thoracic aortas (141) by urea-βME treatment.

(4) Function of Structural Glycoproteins

In the elastic fiber it is hypothesized that the microfibrils play a role in the morphogenesis of the mature elastic fiber. Mature elastic

fibers appear as two morphologically distinct components of a centrally located amorphous structure surrounded by microfibrils 110A° in diameter. In fetal ligamentum nuchae the earliest recognizable elastic fibers appear as a collection of 110A° microfibrils gathered into cylindrical aggregates or in aorta as a flat sheet following the contour of the vessel. The amorphous component which appears later can aggregate between and around the microfibrils taking the form of the microfibrillar template to form a cylindrical fiber in ligamentum nuchae and/or fenestered sheet in aorta.

In a number of other connective tissues immunological studies have indicated that SGP fractions are one of the major tissue and species specific antigenic components (147). Rabbits immunized with corneal SGP reject or opacify rapidly the corneal grafts which would be otherwise well tolerated (136).

Since structural glycoproteins exhibit an insolubility comparable to that of collagen or elastin a structural function seems likely. Dissolved unpurified collagen was found to quickly form mature fibrils in vitro whereas purified collagen aggregates slowly (148). Addition of non-collagenous protein from the connective tissue to purified collagen partially reversed this effect which suggest a role for non-collagenous protein in collagen fibrillogenesis (148). This has been suggested by others (142).

Non-collagenous proteins associated with collagen may have a function during mineralization (150). The close association of phosphoprotein with collagen in bovine dentin has already been noted. Using a combination of biochemical and autoradiographic techniques Weinstock et al (149) traced the synthesis and elaboration of an uncharacterized glycoprotein from within

the odontoblast into the dentin extracellular matrix. The odontoblast is a secretory cell which synthesizes, packages and secretes collagen, GAGs and glycoproteins into the predentin. Glycoprotein, GAGs and collagen (procollagen) are packaged into secretory granules which upon contact with the plasma membrane are discharged into the extracellular matrix. Some of the glycoprotein becomes incorporated into the matrix while some migrates directly to the site of the mineralization front and possibly serves a role during mineralization. Electron microscopic observation of the collagen fibers in dentin (and bone) show them to be coated with an electron dense material whereas the collagen of predentin (before mineralization) does not show this material.

4. Statement of the Problem

Chemical analysis for collagen, proteoglycans and elastin in the insoluble residues of several connective tissues cannot account for their total composition. The presence of a new component; structural glycoprotein (SGP) or acidic structural proteins, is now recognized and has been detected by Robert and others (15, 20, 137, 139, 140, 146) in cartilage, aorta, skin, ligamentum nuchae and rabbit dermis. Structural glycoproteins extracted from these tissues all have similar amino acid compositions. In the case of bovine ligamentum nuchae, acidic proteins have been visualized under the electron microscope as microfibrillar elements closely associated with elastin. Because of the changing proportion of microfibrillar elements to elastin during development of the elastic tissue, it has been proposed by Ross and Bornstein (20) that these microfibrils are involved in the morphogenesis of elastic fibers.

Structural glycoproteins exhibit an insolubility comparable to that of insoluble collagen and elastin and can be solubilized only by reagents which cleave covalent bonds such as alkali, sodium borohydride or other disulfide bond reducing reagents in the presence of denaturing solutes (urea, guanidine HCl). Thus it must be assumed that only subunits of the intact native protein have been obtained in a soluble form.

The purpose of this investigation was directed towards answering the following questions:

(i) Is it possible to separate and purify non-collagenous insoluble proteins from cartilage in their insoluble and presumably native state?

(ii) How do the amino acid composition and solubility properties of the isolated insoluble structural glycoproteins compare to those extracted from other tissues?

(iii) Is the amino acid composition of structural glycoproteins in the insoluble native state the same as that of the solubilized subunits? What physical properties and degree of homogeneity do these solubilized subunits exhibit? How do these properties compare to previously studied extracted structural glycoproteins?

(iv) What is the electron microscopic appearance of structural glycoproteins from cartilage in the native state? Do they differ from that of the microfibrils observed in other connective tissues?

(v) What are the chemical and functional interactions between structural glycoproteins and other extracellular macromolecules such as collagen and proteoglycans?

CHAPTER II PREPARATION OF INSOLUBLE CARTILAGE FRACTIONS

1. Introduction

In recent years a new class of structural component, structural glycoprotein (137), acidic structural protein (15) or acidic protein (20), associated with, but distinct from collagen, elastin and proteoglycans of the acid GAGs, has been recognized in several connective tissues.

Most of the procedures for the isolation of this class of material have revolved around drastic methods such as hot TCA extraction to remove the insoluble collagen (136, 137) followed by solubilization of the remaining material. Recently, milder techniques such as collagenase treatment to remove the insoluble collagen (140, 144) followed by solubilization of the structural glycoprotein (SGP) have been employed. Such solubilized preparations exhibit heterogeneity with respect to solubility (139, 140, 144) and SDS gel electrophoresis (144).

The SGP preparations identified to date have been extracted from calf and rabbit skin, rabbit joint (15) and human aorta (138) among others. The biological function of these materials is not yet known and little data exists on their occurence in cartilage. The objective of the work described in this section was to see if any cartilaginous material remained insoluble after collagenase digestion. Such material, if found, could be compared with extracted SGP from other connective tissues and the relationship to other extracellular connective tissue components could be examined.

2. Materials

(a) Chemical Assays

(i) Hydroxyproline Assay. L-hydroxyproline standard was from Nutritional Biochemicals Corp. (Ohio, USA).

(ii) Glucuronic Acid Assay. Glucuronolactone standard was from Nutritional Biochemicals Corp. (Ohio, USA).

(iii) Hexose Assay. Anthrone and sulfuric acid were from Fisher Scientific Co. (New Jersey, USA), galactose standard from J.T. Baker Chemical Co. (New Jersey USA).

(iv) Protein estimations. Folin Ciocalteu Reagent was from Ingram and Bell (Canada) and crystalline bovine serum standard was from Calbiochem Corp. (California, USA).

(v) Sialic Acid Assay. Sialic acid standard was from Sigma Chemical Co. (Missouri, USA).

(vi) Glucosamine standard for calibration of the amino acid analyses was from Koch-Light (England), galactosamine was from Sigma Chemical Co. (Missouri, USA).

All other reagents used in the assays were ACS grade.

(b) Extraction of Ribs

$CaCl_2 \cdot 2H_2O$ and Hyflo Super Cel were from Fisher Scientific Co.. Guanidine HCl was Grade 1 from Sigma Chemical Co.

(c) Enzyme Digestions

Collagenase Type I (Partially purified) and Collagenase Type III Fr. A

(chromatographically purified) were prepared from Clostridium histolyticum by Sigma Chemical Co. Tris Base was from Sigma Chemical Co., sodium azide from Fisher Scientific Co., and chloretone from K & K Laboratories, (California USA).

(d) Photography

Pictures were taken with a Polaroid 545 Land Camera using Black and White Polapan Type 52 film.

3. Methods

(a) Chemical Assays

(i) Chloride was estimated by the method of Schales and Schales (159).

(ii) Hydroxyproline was determined by the method of Leach (162) or Woessner (161), using L-hydroxyproline as a standard. In the case of the Leach (162) procedure test blanks were performed on duplicate samples by omission of the hydrogen peroxide.

(iii) Glucuronic Acid was determined by the method of Dische (171) or Bitter & Muir (160) using the one-half scale method and glucuronolactone as a standard. When assaying insoluble materials, the sample was dissolved in 1.0N NaOH with heating at 100° x 3 min when necessary. In such cases the standards were treated identically. Test blanks were performed on duplicate samples by omitting the carbazole.

(iv) Hexose was assayed by the method of Yemm and Willis (163) as modified by Andrews et al (133). Galactose was used as a standard and the results for total hexose are expressed in terms of this sugar. Again in the case of assaying insoluble samples, the sample was dissolved in 1.0N NaOH. In such cases the standards were treated identically.

(v) Protein was estimated by the method of Lowry et al (165) using the modification for insoluble proteins (166) when necessary.

(vi) Hexosamines were determined on a Beckman Model 120C Amino Acid Analyser. Freeze dried samples were hydrolysed in 2NHCl at 110^{o} for 6 h in evacuated tubes. The hydrolysates were taken to dryness over NaOH in vacuo, redissolved in water and filtered through a 0.8μ millipore filter (using a Swinney adapter) to remove particles. The filtrate was again taken to dryness, redissolved and assayed.

(b) Extraction of Ribs

Ribs were removed, as soon as possible after death by pentobarbital overdose, from 8-16 week old puppies of undetermined breed. The cartilaginous sections of the ribs between the costo-chondral junctions and the sternebrae were used. After removal of muscle and perichondrium the cartilage was thinly sliced and the slices extracted with 15 times their volume of 2.0M $CaCl_2$ for 24 h at room temperature with gentle stirring (11). After pouring off the supernatant, the process was repeated with 2.5M $CaCl_2$ and the supernatants pooled. For most later preparations 4.0M guanidine HCl was used as an alternative to $CaCl_2$ for extraction (12). The supernatants were clarified

by vacuum filtration with the aid of 5% (w/v) Hyflo Super Cel. The filter cake was sucked dry and discarded without washing. The filtrate was dialysed exhaustively against distilled water and freeze dried. Protein polysaccharide complex (PPC), proteoglycan subunit (PGS) and link glycoprotein (GPL) were prepared according to Hascall and Sajdera (12). The residual slices were then homogenized for 1 h with ten times their volume of distilled water in a Virtis 45 high-speed homogenizer at medium speed. The homogenate was then washed by repeated high speed centrifugation (30,900 - 45,900 x g x 30 min) or exhaustive dialysis against distilled H_2O to bring the Cl^- concentration in the supernatant to less than 0.1m M as detected by the method of Schales and Schales (157).

(c) Effect of Collagenase and Preparation of Insoluble Glycoprotein Fractions

Five procedures were used to investigate the effect of collagenase on the calcium chloride or guanidine HCl extracted ribs (Fraction 1).

(i) Procedure 1

Fraction 1 was suspended (3.0mg/ml) in 0.1M Tris-HCl containing 0.005M $CaCl_2$, 0.02% NaN_3 which was adjusted to pH 7.4. Partially purified collagenase (Type I, 1.5U/ml) was added and the mixture incubated at 37^o for a total time of 23 h. At various time intervals (1h, 2h, 3h, 4h, 23h) the digest was centrifuged at 27,100 x g x 10 min and 1.5ml aliquots were taken from the supernatant for glucuronic acid and hydroxyproline assay. At the end of 23 h the undigested residue was isolated by centrifugation, washed and freeze dried (D_3). The supernatant (containing collagenase

solubilized material) was dialysed by ultrafiltration in an Amicon cell using a XM-50 membrane. The retentate (M.W.>50,000) was freeze dried (D_2). The dialysate was dialysed against a PM-10 membrane, the retentate (M.W. 10,000 - 50,000) freeze dried (D_1) and the dialysate (>10,000 M.W.) discarded.

(ii) Procedure 2

Fraction 1 was suspended (1.5mg/ml) in 0.05 M Tris-HCl containing 0.5M $CaCl_2$, 0.02% NaN_3 which was adjusted to pH 7.4. Purified collagenase (Type III Fr. A, 0.15U/ml) was added and the mixture incubated at 37^{o} for a total time of 23 h. At various time intervals (0.5h, 1h, 2h, 3h, 4h, 5h, 23h) the digest was centrifuged at 27,100 x g x 10 min and 1.5ml aliquots were taken from the supernatant for glucuronic acid and hydroxyproline assay. The pellet was resuspended in the supernatant and the digestion was continued.

(iii) Procedure 3

Fraction 1 was suspended (1.5mg/ml) in the same buffer and digested as in procedure 2. At the various time intervals (0.5h, 1h, 1.5h, 2h) the digest was centrifuged at 27,100 x g x 10 min, the whole supernatant drawn off and assayed for glucuronic acid and hydroxyproline. The pellet was resuspended with fresh enzyme and buffer and the incubation continued.

(iv) Procedures 4 and 5

Procedure 4 is outlined in Figure 1; procedure 5 in Figure 2. Both procedures consist of collagenase (Type III Fr. A) digestion of Fraction 1 followed by a centrifugal separation of the insoluble portions of the digest. The buffer for the digestions was 0.05 M Tris-HCl containing 0.5 M $CaCl_2$,

0.02% NaN_3 adjusted to pH 7.4 at 37°.

For both procedures 4 and 5 the centrifugations were carried out in a Sorvall RC-2 or RC-2B centrifuge at 4° using the SS34 rotor (r_{av}=10.8cm). The isolation of Fraction A and Fraction G from the opaque supernatant is the same in both procedures. Upon centrifugation of the opaque supernatant at high speed (27,000 - 35,000 x g x 20 min), the supernatant is saved and combined with the water wash (see below). The pellet was washed with water (usually 4 times) by resuspension and centrifugation. Upon water washing the pellet resolved into an upper white, fluffy layer (Fraction A) and a bottom yellow, sticky layer (Fraction G). The result of this washing procedure is shown on Figures 3, 4 & 5. The supernatants from the water washing procedure are pooled, dialysed and freeze dried. This fraction is called water wash. After centrifuging the last wash, the top clear supernatant is removed. The fluffy layer (Fr. A) is removed by adding a small aliquot of distilled water, swirling the tube and decanting or drawing off the suspension of Fr. A in a syringe. Fraction G is left as a compact layer at the tube bottom. The separated fractions are then washed several more times (usually four) by repeated centrifugation in distilled water or exhaustive dialysis then freeze dried.

(v) Exhaustive Collagenase Digestion of Fractions A and G

Fraction A or G was suspended (1.0mg/0.9ml) in 0.05M Tris-HCl containing 0.5M $CaCl_2$, 0.1% chloretone which was adjusted to pH 7.4. 0.6U Collangenase (Type III, Fr. A 0.6U/0.1ml buffer) was added and the mixture incubated at 37° with gentle shaking for 24h. Another 0.6U of enzyme (0.6U/0.1ml) was added and the digestion continued for 24h at which time another 0.6U of enzyme was added for a total digestion time of 72h. The digest was centrifuged at 100,000 x g x 30 min, the pellet isolated, washed three times by repeated

centrifugation in distilled water, freeze dried and assayed.

4. Results

(a) Extraction of Ribs

It is clear from Table II that treatment of the rib slices with the 2.0M $CaCl_2$, 2.5M $CaCl_2$ extracts less glucuronic acid containing material than the 85% removal demonstrated by Sajdera and Hascall (11) in bovine nasal cartilage. Water homogenization and subsequent water washing shows that a large proportion of the unextracted glucuronic acid is released during this treatment, however, a considerable portion (38.5%) of the total tissue glucuronic acid remains unextractable from the insoluble portion of the matrix (Fraction 1). The 4.0M guanidine HCl extracted ribs left similar amounts of unextractable glucuronic acid in Fraction 1 (Table II). Fraction 1 prepared by extraction with either salt produced fractions A and G (Table IX) upon collagenase digestion.

(b) Effect of Collagenase & Preparation of Insoluble Glycoprotein Fractions

(i) Procedure 1

A single aliquot of partially purified collagenase (Type 1) was allowed to act on Fraction 1 over a 23h period. Thus the values given in Table III represent the total amount of the components which were solubilized from Fraction 1 at the particular time shown. Table III shows that there is an initial burst in the release of hydroxyproline (collagen) and a non-collagen component (glucuronic acid) during the first hour of digestion, however, with longer incubation times the rate levels off.

Fractionation of the collagenase solubilized components by ultrafiltration (Table IV) shows the low molecular weight material D_1 (10,000 - 50,000) has a high collagen content as well as solubilized non-collagen protein. The high molecular weight material (D_2>50,000) has a high glucuronic acid content and also contains non-collagen protein. Essentially all of the glucuronic acid was recovered in this fraction (Table V). The insoluble residue (D_3) has a low collagen content and contains a high content of non-collagenous protein. Table V shows that this fraction contains little of the total tissue collagen (0.2%) but a considerable portion (26.3%) of the total tissue non-collagen protein.

(ii) Procedure 2

The methodology for the collagenase digestion is similar to that in procedure 1 except that in this case a highly purified collagenase preparation (Type III Fr. A) was used. Again there is an initial burst in the solubilization of collagen and glucuronic acid (Table VI). Approximately one-half of the total hydroxyproline and glucuronic acid was released in the first one-half hour of the 23 h digestion. However, in comparison to procedure 1 (Tables III, V), the rate and total amounts of glucuronic acid and hydroxyproline solubilized from Fraction 1 was lower.

(iii) Procedure 3

Table VII shows that there is an initial burst of solubilization of both components monitored upon first exposure to the enzyme, however, even after repeated exposure to fresh enzyme buffer solution, solubilization

occurs at a more modest rate. The third and fourth additions of fresh enzyme did not effect the same degree of collagen solubilization which was observed upon the first two additions, even though (Table VIII) the insoluble residue still contained a relatively high collagen content. The overall rate of solubilization (at the end of 2 h) of hydroxyproline is greater than in procedure 2 (Table VI), however, the total amounts of hydroxyproline and glucuronic acid solubilized (after 2 h) was less than that using the partially purified collagenase preparation (Table III).

(iv) Procedures 4 and 5

During the execution of the collagenase digestion according to procedure 4 (Figure 1), it was observed after the second or third addition of fresh collagenase that there was a sticky yellow layer on top of the pellet obtained by centrifugation of the digest. Since this material was quite physically distinct from the remainder of the pellet, it was decided to isolate and chemically examine it. The procedure which was found to effectively isolate most of this yellow material was suspension of the whole pellet in water followed by a low speed centrifugation (30 x g x 30 sec). The opaque supernatant was found to contain most of this yellow material since if this opaque supernatant was drawn off and the loose pellet remaining was recentrifuged at high speed (31,000 x g x 10 min) the resultant pellet showed very little of the yellow sticky layer on the top. Further digestion of the loose pellet with collagenase produced more of this yellow sticky material which was isolated in the same fashion and pooled with the previous opaque supernatant. It was fortuitous that repeated centrifugation at high speed in distilled water was chosen (as opposed to dialysis) as a means

to remove the buffer from the opaque supernatant before freeze drying. During this washing procedure another fraction (Fraction A) becomes clearly discernible and appears to evolve from the yellow sticky layer (Fraction G). Both layers (Figure 4, 5) were then isolated as described in the methods and freeze dried.

Since Fraction A sediments with greater and greater difficulty as water washing is repeated, it was considered that there might be some material which did not sediment under the centrifugation conditions used. Thus the water washings were pooled, dialysed and freeze dried.

Procedure 5 (Figure 5) was found to be mechanically simpler than procedure 4 and gives a similar fractionation of insoluble residues. Procedure 5 has been used for all later work, however, three of the preparations in Table IX were obtained by procedure 4. The development of procedure 5 was brought about due to the inability of procedure 4 to effect the isolation of Fractions A and G from some preparations of extracted puppy ribs. In these instances when water was added to the pellet in order to isolate the opaque supernatant, the insoluble residue aggregated into an amorphous material. Upon low speed centrifugation the opaque supernatant was absent. Further investigation of this problem showed that if the pellet from the centrifuged whole digest was suspended in the buffer used for collagenase digestion (and not water) the aggregation was prevented and an opaque supernatant could be isolated. Upon high speed centrifugation of this opaque supernatant, again two insoluble layers could be seen (Figure 6). These two layers were isolated in buffer and assayed for hydroxyproline. The results in Table X show that the hydroxyproline values are much higher.

When the collagenase digestion time was extended as in procedure 5 and applied to the same preparation of Fraction 1, aggregation does not occur. Upon addition of water Fractions A and G were isolated having the compositions shown in Table IX. Using this technique,however, it was observed that the dry weight yield of Fraction A was lower than that obtained previously with procedure 4. This was rectified if the centrifugation force used to spin the digestion mixture was increased to 33,000 - 46,000 x g x 2 h in order to obtain a clear supernatant. It appears that in some preparations the Fraction A tends to stay in the supernatant which reduces its final yield in the pellet.

The analytical data in Table IX show that Fraction 2, A and G are distinctly different materials. Fractions A and G contain much lower hydroxyproline and much higher concentrations of non-collagenous protein than Fractions 1 or 2. In fact some Fraction A and G preparations made subsequent to those shown in Table IX gave zero values for hydroxyproline using the Beckman amino acid analyser and values lower than those in Table IX using the manual procedure (161) (Data not shown). In addition, assays for hydroxylysine on two typical preparations of Fraction A and G gave a range of values from 0 to 0.01% of dry weight.

(v) Exhaustive Collagenase Digestion of Fractions A and G

Table XI shows that exhaustive collagenase treatment sucessfully removes all but trace amounts of collagen from the Fractions A and G.

5. Interim Discussion

The objective of the dissociative extraction procedure with 2.0M $CaCl_2$ or 4M guanidine HCl is to extract soluble components (glycoproteins and proteoglycans) from puppy rib cartilage. This is a preliminary step in the search for insoluble non-collagenous proteins.

After dissociative extraction the rib slices retain their original shape. The observation that water homogenization and subsequent water washing of the homogenate removed an additional amount of glucuronic acid containing material (Table II) suggests that in puppy rib cartilage, a physical disruption of the cartilage extracellular matrix is necessary for more complete removal of proteoglycans. In this respect puppy rib cartilage differs from bovine nasal cartilage where it was shown that there was no difference between the disruptive and dissociative methods with respect to the total amount of tissue glucuronic acid extracted (11). Previous studies on the extraction of proteoglycans from bovine articular cartilage (1) or human articular cartilage (172) also have shown these same dissociative solvents differ sharply in the amounts of proteoglycan extracted from different cartilages.

Table IV gives the chemical composition of Fraction I. Aside from glucuronic acid content, there are other factors which suggest that this fraction may contain non-collagenous proteins. Although Fraction I has a high collagen content, the hydroxyproline content is lower than is found in purified collagens. The hexose content is higher than that found in other vertebrate fibrillar collagens (174). These data are analogous to the analysis on unpurified collagens from a number of other sources (120, 175) where the presence of non-collagenous glycoproteins has been suggested.

Collagenase digestion is an obvious procedure to remove the collagen from this fraction. Collagenase has been used by several workers (2, 16, 85, 110, 140, 144) as a tissue probe to solubilize collagen. The ability to isolate an insoluble non-collagenous protein fraction (D_3) by collagenase digestion (Tables III, IV, V) show that this is a useful approach for the preparation of this connective tissue component. Recovery of non-collagen protein (Table V) was not complete (72.2%, as assessed from the Lowry protein assay on Fractions 1, D_1, D_2 and D_3). This probably was due to non-collagenase proteolytic activity (NCPA) present in the partially pure enzyme preparation which would act on this component to produce peptides (>10,000 M.W.). This soluble fraction was dialysed out according to the methodology used. The presence of most of the glucuronic acid in Fraction D_2 (Table V) indicates that digestion successfully solubilizes most of the non-extractable hexuronic acid. That this fraction has a high molecular weight and contains non-collagenous protein suggests that proteoglycan is being solubilized. Due to the lack of specificity of the enzyme used, it is not possible to comment on the nature of the non-collagenous protein solubilized nor its association to the glucuronate solubilized. The non-collagenous protein could be core protein from proteoglycan or partially degraded insoluble non-collagen protein present in Fraction D_3. Preliminary ultracentrifugation data showed that D_2 has a low S_o value (2.7) compared to extractable proteoglycans. The high collagen content in solubilized Fraction D_1 (Table VI) is presumably due to the presence of collagen peptides solubilized by the digestion. The total recovery (Table V) of this component was low. Again this probably was due to the fact that the digestion produced collagen peptides with a M.W. less than 10,000 and thus discarded. The presence of non-collagen protein in Fraction D_1 could arise from a number of sources. It could be soluble

non-collagen protein not extracted by the dissociative solvents due to physical hindrance or binding to insoluble collagen in the cartilage. Upon degradation of the insoluble network by collagenase these proteins could be released. Alternatively, because of the NCPA the non-collagen protein may be partially degraded core protein from proteoglycan (D_1 contains some glucuronic acid as well) or be degraded non-collagen protein present in Fraction D_3.

Due to the problems arising from the NCPA in the partially purified enzyme preparation, it was decided to use the approach in procedure 1 with purified collagenase (Type III Fr. A) which is essentially free from NCPA (2, 7). This collagenase was used in all subsequent digestions. Although the fractions we are interested in are insoluble, the possibility exists that a partially pure enzyme preparation might 'nick' the insoluble non-collagen proteins or cause some solubilization. The presence of 'nicked' insoluble proteins would make the interpretation of results (Chapters 4 and 5) difficult upon eventual solubilization of these proteins. Francis and Thomas (8) have shown that several proteases will solubilize insoluble structural proteins.

Using procedure 2 there was incomplete (63.1%) solubilization of the total collagen present in Fraction 1 (Table VI). Since a large proportion of both components monitored were released upon initial exposure to the enzyme, procedure 3 was designed in which Fraction 1 is repeatedly exposed to fresh aliquots of enzyme at the given time intervals (Table VII). The objective was to effect a greater and more rapid solubilization of collagen. Over the 2 h period, procedure 3 was moderately more successful than procedure 2 for solubilizing collagen and was one of the factors considered

for development of procedure 4. In that the insoluble residue left after procedure 3 (Table VIII) still contains substantial collagen and non-collagen material, it was rationalized that to obtain insoluble preparations with even lower collagen contents, the collagenase digestion would have to be performed for longer times with higher concentration of enzyme.

Two alternative procedures were found to be necessary to consistently produce two distinct insoluble non-collagen protein fractions. Procedure 5 involves longer collagenase digestion times than procedure 4. This is due to two critical areas in the digestion and fractionation steps. It was found that the digestion time may have to be varied depending upon the starting material (Fraction 1). The digestion must progress to the point where the opaque supernatant (Figure 2) contains 2% hydroxyproline or less (Data not shown), otherwise addition of water to the 33,100 - 46,100 x g x 2 h pellet (Figure 2) causes aggregation rather than separation. If water had been added to the insoluble layers (Figure 6) which had high collagen contents (Table X), the layers would aggregate to form an amorphous material. Binding of non-collagen proteins to collagen has been documented (17, 115). The reason for the variability in collagen digestion is not known. It could be due to the differences in the age of the puppy ribs which varied between 8-16 weeks. For any one complete preparatory scheme (thirteen were carried out on different puppies) there was no way to predict which of procedures 4 or 5 would produce Fractions A and G. In the cases when procedure 4 was not successful, procedure 5 was successful. Batch variations in the collagenase were ruled out as the same batch of enzyme produced Fractions A and G from some puppies but not from others. The second critical point is that in procedure 5 the collagenase digest must be spun at a high enough speed to

obtain a clear supernatant (33,000 - 46,000 x g x h). If not, the yield of Fraction A is low.

After repeated collagenase digestion, the insoluble residues (Fractions A, G and 2 in Table IX) contain only a portion of the total hydroxyproline present in Fraction 1 (1-10% depending on the conditions and length of digestion). This confirms that the bulk of the collagen was solubilized. As well, the bulk of the glucuronate (90-95%), non-collagen hexose* (82-85%) and non-collagen protein (78-87%) have been solubilized. This suggests that these materials (proteoglycans, glycoproteins) are insoluble due to their physical entrapment by the insoluble collagen network. This aspect will be discussed more fully later. Fraction 2 differs the least from Fraction 1, having only a slightly lower hydroxyproline and slightly higher hexose and non-collagen protein concentration. It seems likely that Fraction 2 represents Fraction 1 from which only a part of the collagen has been removed. This interpretation is supported by the observation that the amount of this fraction decreases with increasing number of collagenase digestions. (Data not shown) The presence of a low but significantly different contents of hexose and glucuronic acid in Fraction A and G indicates that they are insoluble non-collagen glycoproteins with tenaciously associated proteoglycan or glycosaminoglycan. This is supported by the fact that the exhaustively digested

* Total hexose corrected for the hexose present in cartilage collagen. This is done by subtracting the hexose content (assuming a value of 4% hexose by weight) in an amount of chick cartilage collagen (35,164) which is equal to the collagen content in the analysed fraction. Inherent is the assumption that the cartilage collagen in the samples contains the same hexose content as the chick cartilage collagen.

Fractions A and G, which contain only trace amounts of hydroxyproline, still contain glucuronic acid and more hexose than can be accounted for by the presence of collagen.

6. Interim Conclusions

A mild selective technique has been devised to isolate two physically distinct insoluble glycoprotein fractions from puppy rib cartilage.

CHAPTER III CHARACTERIZATION OF INSOLUBLE GLYCOPROTEIN FRACTIONS

1. Introduction

The previous chapter described the isolation of two insoluble fractions (A and G) on the basis of their resistance to collagenase and unique physical properties. Their chemical composition indicates that they are primarily non-collagenous glycoprotein residues. Previous studies on extractable structural glycoproteins show that they are solubilized in solvents such as 8M urea (138, 144), 5M guanidine HCl with dithiothreitol (146, 20) and alkali (140, 144). Structural glycoproteins have been characterized by their solubility properties as well as their amino acid composition. This chapter describes studies performed to characterize the insoluble glycoprotein fractions A and G; these include the amino acid composition and those solubility properties which provide some comparison with the solubilized structural glycoprotein preparations of other workers.

2. Materials

(a) Chemical Assays

The materials for the hydroxyproline, Lowry protein, glucuronic acid and hexose assays were the same as described under Materials, Chapter 2. Hydrochloric Acid for amino acid analysis was from Fisher Scientific Co.

(b) Solubility Studies

Sodium hydroxide, urea were from Fisher Scientific Co., dithiothreitol from Calbiochem Corp., tetrasodium ethylenediaminetetraacetic acid was from Sigma Chemical Co.

Sepraphore III Cellulose Polyacetate Electrophoresis strips were from Gelman Instrument Co. (Michigan, USA), Alcian Blue from Matheson, Coleman & Bell (Ohio, USA).

Cupric acetate from J. T. Baker Chemical Co. Chondroitinase AC from Seikagaku Kogyo Co. Ltd., Japan.

Glycosaminoglycan standards were provided by Dr. M. Matthews, University of Chicago, USA.

(c) Photography

The camera and film was the same as that described under Photography, Chapter 2.

3. Methods

(a) Chemical Assays

(i) Estimations of hydroxyproline, glucuronic acid, hexose and protein were carried out by the same procedures described under Methods in Chapter 2.

(ii) Amino Acid Analysis

Amino acid analysis were performed on Fractions A, G and link glycoprotein* (GPL) after hydrolysis of freeze dried samples (stored in vacuo over P_2O_5) in 1 ml 6NHCl containing 2.5 mg phenol at 110° in evacuated tubes for 18, 21 or 24 h. The hydrolysates were taken to dryness over NaOH in vacuo, redissolved in water and filtered through a 0.8 µ millipore filter (using a Swinney adaptor) to remove particles. The filtrate was again taken

* Prepared by the procedure of Sajdera and Hascall (12) from the $CaCl_2$ extracts of the puppy rib cartilage.

to dryness over P_2O_5 in vacuo, redissolved and assayed using a Beckman Model 120C amino acid analyser.

(b) Caesium Chloride Density Gradient Centrifugation

5-10 mg of freeze dried sample was ground with caesium chloride solution (34.1 or 35.5 g CsCl per 100 g solution) in a mortar and pestle. The finely divided suspension was transferred to centrifuge tubes for use in the Beckman SW50.1 (upper part of Figure 7) or SW41 rotors (lower part of Figure 7) and then centrifuged for $2.1 \times 10^6 g_{av} \times h$. At the end of a run densities at the top and bottom of blank (CsCl) tubes and above each visible band of solid material in the test runs were measured using a microlitre pipette as a pycnometer. The bands of insoluble material from the test runs were removed with a needle and syringe for dialysis and analysis.

(c) Solubility Studies

(i) Light Scattering in Sodium Hydroxide.

The OD_{500} of water suspension of Fraction G (1mg/ml) was read against a water blank. In all cases a suspension was created by inverting the cuvette three times immediately before reading the OD_{500}. Additions of sodium hydroxide were made followed by heating (at 100° for 2 min) where indicated. The suspensions were cooled and the OD_{500} again recorded.

(ii) Distribution of Components of Fractions A and G in Sodium Hydroxide.

A suspension (1mg/ml) of either Fraction A or G in 0.02N NaOH was

tumbled for 19 h at 23°. The suspension was centrifuged at 100,000 x g x 30 min. The supernatants were drawn off and the supernatant and pellet assayed for hydroxyproline, Lowry protein, glucuronic acid and hexose.

(1) Identification of Acid Glycosaminoglycans

Fractions A or G were suspended (10mg/ml) in 0.02N NaOH and incubated at room temperature for 19 h. The suspensions were centrifuged at 27,000 x g x 30 min and the supernatants removed. A portion of the supernatant was used for electrophoresis and another portion for chondroitinase AC digestion and subsequent electrophoresis. Electrophoresis of standards and samples on Sephaphore III cellulose polyacetate strips was carried out at 4 ma for 2 h in 0.3M cupric acetate. The strips were stained for 10 min with 1% alcian blue in 1% acetic acid. Destaining was for two five minute periods followed by 20 minute period using fresh 1% acetic acid.

The supernatant was digested for 1 h at 37° with 0.005U chondroitinase AC. Digestion was carried out in 0.04M Tris-acetate buffer pH 7.3. The digestion was repeated with another 0.005U of enzyme. Standards of chondroitin-4-sulfate, hyaluronic acid and heparan sulfate were treated in a similar fashion. The digests were electrophoresed, stained and destained as described above.

(iii) Solubility Studies in 8M Urea

Suspensions (1mg/ml) of Fraction A or G in 8M urea (adjusted to pH 9.0) were tumbled for 19 h or 24 h at 4°. The suspensions were centrifuged at 100,000 g x 30 min. In the case of the 24 h extraction, the pellets were resuspended in fresh urea solutions, tumbled for an additional 24 h and recentrifuged. The pellets were washed three times in water by repeated

centrifugation at 100,000 x g x 30 min, then dissolved in N NaOH and assayed for hydroxyproline, Lowry protein, glucuronic acid and hexose.

(iv) Solubility Studies in 8M Urea with Dithiothreitol (DTT)

Suspensions (1mg/ml) of Fraction A or G were tumbled in air tight centrifuge tubes for 24 h at 4° in 8M urea containing 0.01M dithiothreitol. The solution was prepared by dissolving tetrasodium EDTA (1mg/ml) in 8M urea, adjusting to pH 9.0 with HCl, bubbling nitrogen through the solution for 1 h and then dissolving dithiothreitol to 0.01M. The suspension was centrifuged at 100,000 x g x 30 min at 4°. The pellets were resuspended in fresh urea - DTT solutions and tumbled for an additional 24 h. The pellets were washed three times in water by repeated centrifugation at 100,000 x g x 30 min, dissolved in N NaOH and assayed for hydroxyproline, Lowry protein, glucuronic acid and hexose.

4. Results

(a) Amino Acid Composition

Table XVII shows that Fractions A, G and link glycoprotein (GPL) contain a high content of acidic amino acids (200-250 residues/1000 residues). Both Fractions A and G have similar amino acid compositions, but they differ significantly from each other in their contents of lysine, aspartate, glutamate, proline, alanine and isoleucine. The ratio of aspartate/glutamate is significantly higher in Fraction A than in Fraction G. Although the half cysteine values are approximate, both fractions have a high content of cysteine. The link glycoprotein (GPL) prepared from puppy rib cartilage has an amino acid composition which resembles GPL from bovine nasal cartilage (12).

Fraction A resembles link glycoprotein in most of the amino acids but differs in tyrosine, phenylalanine and alanine. Both Fraction A and GPL have a high ratio of aspartate/glutamate amino acids (>1).

(b) Caesium Chloride Density Gradient Centrifugation

Tubes containing 34.1g CsCl per 100g solution gave a gradient of 1.10 - 1.39g/ml and those containing 35.5g CsCl per 100g solution gave a gradient of 1.20 - 1.41g/ml at the end of the centrifugation run. As shown in Figure 7, Fraction A formed a single band at the bottom of the tube when 34.1g CsCl/100g soltuion was used as the loading solution and above the bottom when a 35.5g CsCl/100g solution was used. The bands formed in the density range 1.30 - 1.38. Fraction G formed a single band in the range 1.20 - 1.28g/ml with either 34.1 or 35.5g CsCl/100g (not shown) as the loading concentration.

(c) Solubility Studies

(i) Light Scattering in NaOH

Light scattering was used to follow the solubilization of Fraction G in NaOH. Figures 8, 9, and 10 show that there is a gradual solubilization of insoluble material with increasing amounts of sodium hydroxide. The use of heat (Figure 8, 9) increases the rate of solubilization, but does not solubilize any more G than is dissolved at room temperature with longer times (Figure 10).

(ii) Distribution of Components of Fraction A and G in 0.02N NaOH

Tables XII and XIII show the effect of alkali at room temperature on the various components present in Fractions A and G. For both fractions 0.02N NaOH does not solubilize the minor collagen component (as monitored by hydroxyproline), but it is effective in solubilizing 40-50% of the non-collagen Lowry protein. All of the glucuronic acid is solubilized in Fraction A and most (75%) solubilized in Fraction G. The effect of alkali on the hexose component of the two fractions is different. In Fraction A about 50% is solubilized, whereas in Fraction G most remains insoluble.

(1) Identification of Acid Glycosaminoglycans

The glycosaminoglycans extracted with alkali from both A and G ran as a single spot (Figure 11) with the mobility of the chondroitin-4-sulfate standard. Upon chondroitinase AC digestion, which digests chondroitin 4 and 6 sulfate, the spots disappeared. (Results not shown)

(iii) Solubility Studies in 8M Urea

8M urea alone caused no marked solubilization of any of the components monitored for Fraction A (Table XIV) or G (Table XV).

(iv) Solubility Studies in 8M Urea with Dithiothreitol

In contrast to the effect of 8M urea alone, inclusion of 0.01M DTT into the solvent caused an increase in the solubilization of some of the components in Fractions A and G (Table XVI). For both fractions much more non-collagen Lowry protein and glucuronic acid are solubilized than with 8M

urea alone. There is little effect on the collagen component (hydroxyproline values) where the results are similar to 8M urea alone. This is expected as collagen is known not to contain cysteine.

5. Interim Discussion and Conclusions

The objective for scrutinizing the degree of solubilization with increasing sodium hydroxide concentration (Figures 8, 9, 10) was to see if there were any breaks in the solubilization curve which might indicate the number of distinct dissolving components. Since no clear-cut breaks were obtained this type of experiment was abandoned.

Tables XII and XIII show that solubilization of non-collagen components does occur at low alkali concentrations. Most notable was the good solubilization of glucuronic acid which allowed identification of the glycosaminoglycans as chondroitin-4 or 6-sulfate. Some preparations of Fractions A and G were dissolved completely in 0.2N NaOH by heating for 0.5 min at 100° but others were not. It is extremely difficult to evaluate whether the effect of dilute alkali is by disaggregation and solubilization or chemical cleavage of covalent bonds. Thus the use of this solvent as a probe for characterization of these insoluble residues was discontinued. The fact that chondroitin-4 or 6-sulfate is released by dilute alkali suggests chemical cleavage of O-glycosidic linkages involving serine or threonine as these bonds have been shown to be alkali labile (42). It appears that the bonds in Fraction A and Fraction G are somewhat more alkali labile as Partridge and Elsden (42) found the minimum conditions for cleavage of the O-serine

glycosidic linkage (in bovine nasal cartilage) to be 0.1N NaOH at 25° for 20 h. A similar linkage (in shark cartilage) was stable in 0.05N NaOH at 25° for 72 h (93).

The other results will be discussed more fully in Chapter 6. However, at this stage three interim conclusions were drawn:

(i) The amino acid composition of both Fractions A and G broadly resembles that of the solubilized structural glycoprotein fractions of other workers (15, 137, 139, 146).

(ii) The insoluble A and G preparations differ in density and in their content of some amino acids.

(iii) The use of reducing agents known to produce subunits by splitting disulfide bonds in other proteins is a promising procedure for solubilizing Fractions A and G.

CHAPTER IV CHARACTERIZATION OF SOLUBILIZED GLYCOPROTEIN FRACTIONS A AND G

1. Introduction

The results in Chapter 3 indicate that the insoluble Fractions A and G are soluble in some of the solvents which have been used to solubilize structural glycoproteins from other connective tissues (20, 138, 144, 146). It was thought that further characterization of the insoluble fractions A and G must involve their solubilization, preferably by a non-destructive technique. This would allow more specific comparisons to the extracted structural glycoproteins from other connective tissues and provide a further insight into the chemical and physical properties of A and G. The data presented in this chapter represent a beginning in the further characterization of Fractions A and G following a treatment which solubilizes most of these insoluble residues.

2. Materials

(a) Chemical Assays

The materials for the hydroxyproline, Lowry protein, hexose and amino acid analysis were the same as that described under Materials, Chapters 2 and 3.

(b) Reduction and Alkylation of Fractions A and G

Sodium iodoacetate was purchased from Sigma Chemical Co. 1-^{14}C Iodoacetic acid had a specific activity of 0.07m Ci/mg dry weight and was purchased from New England Nuclear (Mass. USA). Dialysis tubing with a molecular weight cutoff of 3500 was from A.H. Thomas Co. (Pa. USA).

PCS solubilizer was from Amersham-Searle (Ill. USA), xylene from J. T.

Baker and Standard ^{14}C toluene from New England Nuclear.

(c) Solubilization of Reduced and Alkylated Fractions A and G

Disodium hydrogen phosphate and sodium dihydrogen phosphate were from J. T. Baker. Sodium dodecyl sulfate was from Fisher Scientific Co. or Dupont (Canada).

(d) Gel Chromatography

Sephadex G-200 was from Pharmacia (Sweden), Dowex 1-X2 from Sigma Chemical Co.

(e) Sodium Dodecylsulfate (SDS) Polyacrylamide Gel Electrophoresis

Acrylamide and N, N'-methylenebisacrylamide were from Eastman Kodak Co. (N.Y., USA), ammonium persulfate, tetramethylethylenediamine, glycine, β-mercaptoethanol, coomassie blue, horse heart cytochrome C were from Sigma Chemical Co., bromophenol blue from Matheson, Coleman & Bell, trichloroacetic acid, isopropanol, isobutanol from Fisher Scientific Co., acetic acid from J. T. Baker Co., polyacrylamide gradient slabs from Isolab (Ohio, USA), ovalbumin from Mann Research Labs (N.Y. USA) and NCS Tissue Solubilizer from Amersham-Searle.

(f) Photography

Black and white photographs were taken with the same film and camera described in Chapter 2.

Colour prints were taken with Type 58 film, using a Wratten 80B filter.

3. Methods

(a)Chemical Assays

Quantitation of protein and amino acid analysis were performed as described in Chapters 2 and 3.

(b) Reduction and Alkylation of Fractions A and G

The reduction and alkylation procedure of Hudson and Spiro (167) was followed except that dithiothreitol in 8M urea containing 1mg/ml Na_4EDTA was used instead of β-mercaptoethanol and the total reduction time was 48 h. The second addition of dithiothreitol was made after 24 h. Alkylation was followed by dialysis and freeze drying the retentate. For the preparation of ^{14}C labelled Fractions A and G one of two procedures was used:

(i) Method 1

After reduction for 48 h the fractions were dialysed in cellophane tubing against an appropriate volume of 0.5M Tris-HCl buffer, (pH 8.5 containing 8M urea, 1mg/ml Na_4 EDTA)in order to bring the dithiothreitol concentration down to a 3 fold molar excess over the calculated sulfhydryl groups in the protein. The dialysis was carried out in evacuated Buchner flasks. After dialysis, alkylation was performed with 1-^{14}C-Iodoacetic acid at a molar ratio to the calculated sulfhydryl groups of 1.1:1 according to the procedure of Hudson and Spiro (167).

(ii) Method 2

After reduction for 48 h the fractions were dialysed exhaustively against 0.5M Tris-HCl buffer (pH 8.5 containing 8.0M urea, 1mg/ml Na_4 EDTA) in a nitrogen pressurized Amicon cell using a PM-10 membrane. After dialysis,

alkylation was performed with 1-^{14}C-Iodoacetic acid at a molar ratio to the calculated sulfhydryl groups of 1.1:1 according to the procedure of Hudson and Spiro (167).

Radioactive samples were counted in a Beckman LS-250 Liquid Scintillation counter. The scintillant used was a 2:1 mixture of PCS solubilizer; Xylene as recommended by the manufacturer. Insoluble samples were counted after dissolution in 0.5ml N NaOH, addition of 10ml of cocktail and 1ml water. Radioactive counts were corrected for quenching by the addition of internal standard (^{14}C-toluene) where indicated.

(c) Solubilization of Reduced and Alkylated Fractions A and G

Solubility of reduced and alkylated Fractions A and G was tested in 0.1M phosphate buffer, (pH 7.0) containing 0.02% NaN_3 and one of: 0.1% SDS, 1.0% SDS, 5.0% SDS, 8M urea. The lyophilized fractions were solubilized by suspension (approximately 125 µg/ml) and tumbling for 24 h at 23°. After centrifugation at 80,000 x g x 30 min the supernatants were drawn off, counted and corrected for quenching by the addition of ^{14}C-toluene internal standard. The final cocktail consisted of 0.5ml of supernatant, 1ml water and 10ml PCS:Xylene (2:1).

(d) Gel Chromatography

Solubilization of reduced and alkylated Fractions A and G for gel chromatography was effected by suspension (4mg/ml) and tumbling for 48 h at 37° in 5.0% sodium dodecylsulfate containing 0.1M phosphate buffer (pH 7.0), 0.02% NaN_3. After centrifugation at 80,000 x g x 30 min at 23° to remove small amounts of insoluble material, the supernatants were dialysed

against water in which Dowex 1-X2 was suspended to remove free sodium dodecylsulfate. The retentate was freeze dried and kept for gel chromatography and analytical ultracentrifugation. The pellets were washed four times with distilled water by repeated centrifugation at 80,000 x g x 30 min at 23° and dried in vacuo over P_2O_5. The supernatants and pellets were assayed for Lowry protein, hydroxyproline, hexose and the total amino acid composition as described in Chapter 2.

Gel chromatography was performed on Sephadex G-200 in a 2.5 x 37 cm column. Elution was achieved with the buffers indicated in Figures 12-16 at a hydrostatic head of 20cm and a flow rate of 10ml/h. Sample volumes were 2-3 ml and 4.3 ml fractions were collected. In the cases where isolated peaks were rechromatographed, the fractions under the peak were pooled, concentrated in an Amicon cell (PM-10 membrane), then dialysed against the buffer in which the column is run. For the radioactive profiles, 0.5ml of each fraction was mixed with 0.5ml water and 10ml of PCS:Xylene (2:1). Counts were corrected for background but not for quenching.

(e) SDS Gel Electrophoresis

Solubilization of Fractions A and G for electrophoresis was similar to that described in solubilization for gel chromatography. Several procedures were used to explore the behaviour of Fractions A and G on SDS polyacrylamide gels. For the electrophoresis of A or G fractionated by gel chromatography, the fractions grouped into distinct peaks were pooled, assayed by the Lowry procedure (165), concentrated in an Amicon Cell (PM-10 membrane) and equilibrated in a dialysis tube with 0.062M Tris-HCl buffer

(pH 6.7) containing 5.0% SDS before electrophoresis.

(i) Procedure A

SDS polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (168). The running gels contained 5% acrylamide and twice the concentration of the bisacrylamide recommended for the 10% acrylamide gels. The gels were fixed in 50% TCA for 16 h, stained with 0.25% coomassie blue in 20% TCA for 2.5 h and destained in 7% acetic acid.

(ii) Procedure B

Disc gel electrophoresis was carried out on 2.5, 3.75 and 5.0% polyacrylamide running gels which were made up in 0.375M Tris-HCl buffer (pH 8.9) containing 0.1% SDS. The 5% gels were cast as described by Hudson and Spiro (167). The 2.5% and 3.75% polyacrylamide gels contained 0.26% bisacrylamide and 0.203% bisacrylamide respectively. The electrode buffer was 0.005M Tris, 0.038M glycine buffer (pH 8.3) containing 0.1% sodium dodecy-sulfate. Tests and standards containing 30-50µg protein in 20-50µl of electrode buffer with 1% SDS were mixed with an equal volume of 8M urea and incubated for 1-2 h at room temperature with 10µl β-mercaptoethanol and 3µl bromophenol blue. They were then layered over the running gel and electrophoresis carried out at 3mA/tube until the dye had penetrated the running gel, then at 5mA/tube until the tracking dye was within 1 cm of the bottom of the gel. The dye fronts were marked with India ink. The gels were fixed in 50% TCA overnight, stained with 0.25% coomassie blue in 20% TCA for 2.5 h and destained with 7% acetic acid.

(iii) Procedure C

Gel electrophoresis was performed on polyacrylamide gradient slabs (2.5 - 27% polyacrylamide gradient). The slabs were equilibrated in 0.005M Tris, 0.0382M glycine buffer (pH 8.3) containing 1% SDS, by pre-running the gels in the buffer alone until the buffer front had passed through the gels. Samples containing 25µg protein, dissolved in 25µl running buffer were mixed with an equal volume of 8M urea and incubated at room temperature with 10µl β-mercaptoethanol for 1 - 2 h. Electrophoresis was carried out at 3mA until the cytochrome c standard had stopped migrating. The slabs were fixed and stained by the procedure of Fairbanks et al (169).

(iv) Procedure D

SDS gel electrophoresis was carried out by a procedure similar to that of Laemmli (170) except that the stacking gels were made up in 0.062M Tris-HCl buffer, pH 6.7; the running gels were made up in 0.364M Tris-HCl, pH 8.9 and both gel buffers contained SDS at the various concentrations specified in Figures 20, 23-30. The stacking gels contained 2.5% polyacrylamide and acrylamide/bis=$\frac{9.3}{1}$. The running gels contained 8, 10 or 12% polyacrylamide and acrylamide/bis=$\frac{37}{1}$. The final concentration of TEMED in the gels was 0.05% and of ammonium persulfate (added last) 0.075%. In the running gels which contained urea, the urea was dissolved in the ammonium persulfate solution such that the final concentration of urea in the running gel was 5M. The electrode buffer was 0.005M Tris, 0.0382M glycine, pH 8.3 containing the same concentration of SDS as the gel buffer. Samples, not fractionated on Sephadex G-200, and standards, containing 30-50µg protein in 25-50µl of stacking gel buffer with 1.0% SDS, were mixed with an equal volume of 8M urea and incubated for 2 h at room

temperature with 10 µl β-mercaptoethanol and 3 µl bromophenol blue. They were then layered on top of the sample gel. Electrophoresis was carried out at 3 mA per gel tube until the tracking dye entered the running gel and then at 6 mA per tube until the bromophenol blue was within 1 cm of the bottom of the gel (about 1 h). Fixation and staining of the gels was carried out by the procedure of Fairbanks, Steck and Wallach (169) using coomassie blue.

For the ^{14}C profiles (Figures 21, 22) the electrophoresis gels were sliced with a razor blade and each slice placed in a scintillation vial containing 0.5 ml of an NCS:H_2O mixture (9:1). The vials were heated at 50° for 2 h to leach out the radioactivity then cooled to room temperature. Ten ml of PCS:xylene (2:1) scintillant was added to the vials which were allowed to dark adapt for 24 h. Radioactivity was counted and corrected for quenching.

(f) Analytical Ultracentrifugation

Solubilization of Fractions A and G was as described in solubilization for gel chromatography. The lyophilized, SDS treated fractions were dissolved in 0.1 m phosphate buffer, pH 7.0 containing 0.1% SDS, 0.02% NaN_3 to produce the concentrations shown in Figures 31, 32. Sedimentation velocity measurements were made using a Beckman Model E analytical ultracentrifuge. Runs were performed at 38° in order to maintain complete solubilization at the concentrations necessary to obtain a Schlieren pattern. Sedimentation values were calculated for each concentration.

4. Results

(a) Reduction and Alkylation of Fractions A and G

Good recoveries of Fractions A and G were achieved during the reduction and alkylation procedure (Table XVIII). For the preparation of ^{14}C-labelled fractions Method 2 was found to give material with higher specific activities but the percentage recovery was less that that using Method 1 (data not shown). The amino acid analysis (Table XIX) of Fractions A and G after reduction and alkylation show some differences to those reported (Table XVII) before treatment. The composition of Fraction A changed the least. The amino acid analysis (Table XXIV) of the SDS soluble reduced and alkylated fractions show some differences to those reported in Table XVII, and Table XIX. In that only single samples of the reduced and alkylated fractions were analysed, statistical comparisons cannot be made and not much weight can be attached to these results. Further work is required to establish whether the differences in amino acid contents of the insoluble A and G do decrease after reduction, alkylation and solubilization.

(b) Solubilization of Reduced and Alkylated Fractions A and G

Of the solvents tested, 0.1M phosphate buffer (pH 7.0), 0.02% NaN_3 containing 5.0% SDS was judged to be the best for solubilization of the labelled protein in Fractions A and G (Tables XX, XXI). The data were obtained using dilute (125µg/ml) suspensions of material. In that monitoring the solubilization of label is not necessarily an indication of the effect of the solvent on all the protein in the fractions, the distribution of Lowry protein was also examined (Table XXII). In this experiment, suspen-

sions (4mg/ml) were used which upon solubilization give more appropriate concentrations for use in gel chromatography and electrophoresis. Table XXII shows good solubilization and recovery of Fractions A and G with 5% SDS in 0.1M phosphate buffer at higher solute/solvent ratios. Because of the difficulty of removing sodium dodecylsulfate bound to protein, the analytical data on the SDS solubilized Fractions A and G is not as extensive as for the insoluble fractions shown in Table IX. However, the data in Table XXIII indicates that the material solubilized by reduction, alkylation and extraction with sodium dodecylsulfate closely resembles the non-collagenous glycoprotein portion of Fraction A and G, while a minor collagneous component remains insoluble.

(c) Gel Chromatography

Gel chromatography of alkylated SDS-solubilized Fractions A and G show that both exhibit a high (Peak I) and a low (Peak III) molecular weight peak (Figure 12). The ^{14}C profile indicates an intermediate peak (Peak II) between peaks I and III (Figures 13, 14). Recovery of Lowry protein from the columns is 80-90%. Upon concentration and rechromatography of Peak II, Fraction A (Figure 16) the peak appeared not to redistribute in the column and the greatest proportion eluted in the same position as in the original chromatogram. The broad peak obtained is possibly due to diffusion, however, the possibility exists that there are some aggregates in the high

molecular weight region of the peak and some smaller subunits present in the low molecular weight region. The only bit of evidence that suggests there may be redistribution on Sephadex was that rechromatography of Peak 1, Fraction A in 0.1% SDS (Figure 15) produced a slight shift in the elution pattern.

(d) SDS Gel Electrophoresis

The first attempts to characterize reduced, alkylated SDS soluble Fractions A and G electrophoretically were made using the Weber and Osborn procedure (168). Figure 17 shows the results which are typical of several attempts using this system. Neither Fraction A nor G were resolved with distinct bands. The range of sizes indicated by the stained area on the A and G gels is compatible with Sephadex G-200 gel chromatography profiles.

Due to the lack of definitive results with the Weber and Osborn system, the SDS system described in procedure B was used. Figure 18 illustrates the results obtained using different polyacrylamide concentrations. With the 2.5% polyacrylamide gels the pore size is too large to be capable of separating any of the standard proteins which range between 17,000 to 450,000 in molecular weight. On these gels both Fractions A and G exhibit a very heavy band running in the same region as the standards. As the polyacrylamide concentration increases, the resolving power of the gels increase and the standards begin to be separated from each other. Fractions A and G still appear to be running with the low molecular weight standard (myoglobin), however, the intensity of the bands diminish. In the 3.75 and 5.0% polyacrylamide gels, there is no evidence of a high molecular weight band (i.e. between apoferritin and BSA) corresponding to either of Peaks I or II

the Sephadex G-200 profiles.

In that these results again were not conclusive, procedure 3 using a polyacrylamide gradient was used. Figure 19 shows some of the results of these studies. Standard cytochrome c (M.W. 12,500) gives one major and one minor band, but with both A and G there is a continous smearing. This is similar to the results obtained using the Weber and Osborn system (Figure 17). Peak III from the Sephadex G-200 profile of Fraction G exhibits a similar behaviour except that the smearing does not start at as low a polyacrylamide concentration which suggests this material has a lower molecular weight.

Procedure D was designed to obtain more information about the electrophoretic properties of Fractions A and G. This procedure was most successful when utilized with different SDS and polyacrylamide concentrations. Fractions A and G (Figure 20) in 0.1% SDS exhibit a predominant small molecular weight component which runs with a similar electrophoretic mobility to cytochrome c on the 8% polyacrylamide gels. As the polyacrylamide concentrations increases to 12%, Fractions A and G appear to aggregate. This is indicated by the more lightly stained bands in the region of cytochrome c and the presence of more stained material at the tops of the gels. The 12% polyacrylamide gels resemble most closely the Sephadex G-200 profile showing a high molecular weight component and a low molecular weight one.

Electrophoresis under the same conditions except in 1.0% SDS produces a dramatic change in the mobility of Fractions A and G (Figure 20). Under these conditions both fractions run near the ovalbumin standard (M.W. 43,000). Again aggregation of Fractions A and G is induced at higher polyacrylamide concentrations. On the 12% gels, the material running with ovalbumin is barely visible and more staining material appears at the top of the gels. The ^{14}C profiles (Figures 21, 22) support the observation that increasing

the polyacrylamide concentration effects aggregation of these fractions. The use of urea (5M) in the polyacrylamide running gels did not have any effect on the electrophoretic behaviour of Fractions A and G. Again both fractions ran with ovalbumin in 1.0% SDS and aggregated at the top of the gels at the higher polyacrylamide concentrations (Figure 23).

In order to see if the observed electrophoretic behaviour was peculiar to the unfractionated A and G, the Sephadex G-200 peaks from Fractions A and G were electrophoresed under similar conditions (Figures 23-30). Peaks I, II, III from Fraction A ran similarily in 0.1% SDS in that on 8% polyacrylamide all three peaks exhibited a predominant small molecular weight band which runs between ovalbumin and cytochrome c. With increasing polyacrylamide concentration all three peaks aggregate and hang up at the top of the gels, however, some intermediate bands can be seen. In the presence of 1.0% SDS, Peak II Fraction A (Figure 25), I and III (not shown) ran with the mobility of ovalbumin on the 8% polyacrylamdie gels. On 10% and 12% gels aggregation occurs and the samples remain at the tops of the gels.

The Sephadex G-200 peaks I, II, III from Fraction G (Figure 25-30) exhibit essentially the same behaviour as those from Fraction A with respect to polyacrylamide and sodium dodecylsulfate concentration but with the following exception. Peak I from Fraction G runs slightly behind ovalbumin on 8% polyacrylamide under all the conditions tested (Figures 26, 27, 28) however, as before, increasing the polyacrylamide concentration induces aggregation (Figure 26 28). This polyacrylamide-induced aggregation is unlike that observed with unfractionated A and G (Figure 23). Peak I, Fraction G in 0.1% SDS, 5M urea (Figure 27) moves closer and closer to cytochrome c with increasing acrylamide concentration. The use of heat during the preincubation period does not have any effect on the mobility of this peak. Elevation of the SDS concentration from 1% to 5% (Figure 29)

also does not have any effect on the mobility of peak I.

(e) Analytical Ultracentrifugation

As shown in Figures 31, 32, there was a very poor or no relationship between sedimentation velocity and concentration thus preventing meaningful extrapolation to zero concentration. As a result, no attempts were made to calculate the molecular weight of Fractions A or G using the technique of Tanford et al (176) for rigorous determination of the molecular weights of proteins (glycoproteins) in the presence of detergents. It is notable that both A and G sedimented as single symmetrical peaks (Figure 33, a, b). When both fractions were run under identical conditions (Figure 33c) the calculated S values were different.

5. Interim Discussion and Conclusions

After reduction, alkylation and extraction with SDS 82-87% of the protein is solubilized. Gel electrophoresis of solubilized A and G shows the presence of either one or two bands and gel chromatography shows both high and low molecular weight peaks. Analytical ultracentrifugation shows a single molecular weight species. The production of a low molecular weight electrophoresis band from the high molecular weight sephadex fraction indicates that there is aggregation and disaggregation of subunits in SDS.

CHAPTER V FURTHER CHARACTERIZATION OF INSOLUBLE GLYCOPROTEIN FRACTIONS

1. Introduction

Chemical analysis of insoluble glycoprotein fractions A and G (Table IX) indicates that each glycoprotein fraction contains residual collagen and proteoglycan (or acid glycosaminoglycan).

The two objectives of the work described in this chapter were: -

(a) to define the nature and binding of the residual glucuronate containing material in the insoluble residues

(b) to examine by electron microscopy the ultrastructure of A and G and their relationship to collagen.

2. Materials

(a) Chemical Analysis and Extraction Procedures

Materials for the glucuronic acid assay were the same as described previously. Guanidine HCl was Grade I from Sigma Chemical, sodium acetate was from Fisher Scientific Co.

(b) Electron Microscopy

Phosphotungstic acid was from Sigma Chemical Co., Alcian Blue from Matheson, Coleman & Bell, ruthenium red from British Drug Houses (England), collidine from Eastman Kodak Co. and lead acetate from Fisher Scientific Co.

3. Methods

(a) Chemical Assays

Estimation of glucuronic acid was carried out as described in Chapter 2.

(b) Extraction of Fractions A and G with Guanidine HCl

Fraction A or G was suspended (1mg/ml) in 4M guanidine HCl containing 0.05M acetate adjusted to pH 5.8. The suspension was tumbled at room temperature for 24 h and spun at 80,000 x g x 30 min. The supernatant was drawn off, dialysed against distilled water and concentrated by ultrafiltration using a UM-2 membrane. Pellets and supernatants were assayed for glucuronic acid.

(c) Extraction of Fractions A and G with Sodium Acetate

Fraction A or G was suspended (1mg/ml) in 0.5M sodium acetate adjusted to pH 5.8. The suspensions were tumbled at room temperature for 2 h and spun at 80,000 x g x 30 min. The supernatant was drawn off. The pellet was resuspended and extracted twice more as described above. The supernatants were combined, concentrated by ultrafiltration with a UM-05 membrane and assayed for glucuronic acid.

(d) Electron Microscopy

Insoluble Fractions A or G were suspended in water (0.25mg/ml) and sonicated (2 x 15 sec) at 0°. Proteoglycan (from bovine nasal septum) was added to some preparations at a concentration of 0.1mg/ml. A drop (about 1 µl) of each preparation was placed on a carbon coated copper grid (200 or 400 mesh) and the water allowed to evaporate at room temperature. The dry grids were stained by floating them on successive drops of stain or wash placed on dental wax. Four different procedures were used:

(i) PTA. 2 min on 1% phosphotungstic acid (pH 5.6) followed by two 1 min washes with water.

(ii) Alcian + PTA. 1 min on 1% Alcian Blue in 5% acetic acid followed by two 1 min washes with water and then by 2 min on 1% phosphotungstic acid (pH 5.6) and two further washes with water.

(iii) Ruthenium red. 20 min on a solution of 1mg/ml ruthenium red (centrifuged at 10,000 g for 20 min) in 0.05M collidine-HCl buffer (pH 7.2), followed by two 2 min washes with water.

(iv) Lead. 10 min on a saturated solution of lead hydroxide, prepared by the method of Millonig (177) or on 2% lead acetate in 0.05M sodium acetate-acetic acid buffer, pH 5.8, followed by two 2 min washes with water.

4. Interim Results and Discussion

(a) Extraction with Guanidine HCl and Sodium Acetate

Both solvents were successful in removing some (but not all) of the residual glucuronic acid, (Tables XXV, XXVI). In that guanidine HCl and sodium acetate are not known to cleave covalent bonds, at least one-half of the glucuronic acid containing material in Fractions A and G is held in a non-covalent association. 4.0M guanidine HCl has been used to extract proteoglycans from bovine nasal cartilage (12). 0.5M sodium acetate, pH 5.8 has been used to study the interaction of proteoglycan core protein with hyaluronic acid (78).

(b) Electron Microscopy

Electron microscopy shows that both insoluble glycoprotein fractions stain with lead, ruthenium red or alcian blue plus phosphotungstate and that G contains many fine filaments. Material with the same appearance and staining properties was found to occur on the surface of collagen fibers in the undigested cartilage residue. A detailed discussion of the electron micrographs appears on pp. 74-77.

CHAPTER VI GENERAL DISCUSSION AND CONCLUSIONS

Using mild, selective techniques of dissociative salt extraction to remove soluble components, followed by collagenase digestion to remove insoluble collagen, two insoluble glycoprotein fractions (A and G) have been isolated. Thus the first objective formulated on page under "Statement of the Problem" has been achieved. The significance of these preparations is discussed in detail below under a number of separate headings.

Homogeneity of the insoluble preparations.

Fractions A and G were examined in the insoluble state by density gradient centrifugation in caesium chloride. The finding that A and G with low but approximately equal hydroxyproline contents (less than 1%) give essentially single bands with different densities shows that the non-collagenous portions of the two (94% or more of the total) are distinct insoluble entities though the possibility that one or both type of particle contains more than one type of molecule is not eliminated by this observation. Further evidence concerning homogeneity was only obtainable with sub-units prepared by reduction, alkylation and SDS solubilization.

The non-collagen protein contents (Table IX) of the two fractions are similar. It is tempting to speculate that the difference in buoyant density between A and G is due to the differences in their proteoglycan contents. Fraction A has a higher content of proteoglycan than does Fraction G and has a higher buoyant density. This is consistent with the observations of Hascall et al (12) and Heinegard (178). It may be noted that Anderson et al (17) found that two solubilized glycoprotein fractions

could be separated by density gradient centrifugation from extracts of bovine tendon.

Relationship between Fractions A and G and other connective tissue proteins.

It may be noted that there is a broad similarity between the amino acid compositions of Fractions A and G (Table VIII) and previously published data for the solubilized structural glycoproteins (2, 19, 137, 139). In particular, they all show a high content of aspartic and glutamic amino acids and they clearly differ from collagen, elastin and core protein of proteoglycan in their content of these and other amino acids.

Table XVI shows that the insoluble glycoprotein Fractions A and G differ significantly from each other in their content of five amino acids and the molar ratio aspartate/glutamate is significantly higher in Fraction A than in G. In all connective tissues where amino acid analyses have been reported for unfractionated SGP fractions aspartate/glutamate is equal to, or less than 1 (15, 19, 137, 138). In contrast, the soluble glycoprotein associated with proteoglycan aggregates in cartilage (GPL) has a ratio aspartate/glutamate which is greater than 1 (12). This cannot be due to contamination with the core protein of the proteoglycan, since aspartate/glutamate is lower than 1 in this material (12). GPL prepared from puppy rib cartilage (Table XVII) was found to have an amino acid composition closely resembling GPL from bovine nasal cartilage. Fraction A resembles GPL in most of the amino acids, but differs in tyrosine, phenylalanine and alanine. The conclusions from this comparison of amino acid analyses are that GPL, A and G each contain a distinct protein portion, although it

is quite possible at this stage that each is a mixture containing both a common and a unique protein.

Glycoprotein Fractions A and G exhibit solubility properties which are distinct from plasma glycoproteins. The solubility properties of A and G are similar to previously studied structural glycoproteins (15, 137, 144) with the exception of their poor solubility in 8M urea solutions. The reason for this difference is not known. As with previously studied SGP (15, 144) alkali was a better solubilizing agent than 8M urea (Tables XII-XIV). That A and G are effectively solubilized by 8M urea-DTT solutions lends support to the work of Furthmayer and Timpl (140) which indicated that disulfide linkages were involved in maintaining the insolubility of SGP in connective tissues.

Homogeneity and properties of solubilized A and G.

After reduction, alkylation and extraction with 5% SDS (Tables XVIII, XXII) 80-85% of the protein in Fractions A or G was recovered in the soluble form. The solubilized material contains only trace amounts of hydroxyproline, (Table XXIII) and thus is essentially collagen free. Differences in amino acid composition of A and G before and after reduction, alkylation and SDS treatment were observed (Tables XVII, XIX, XXIV). It may be that iodoacetic acid is reacting with other amino acids (i.e. His, Lys, Met),(179, 180) which produces derivatives which are not detected by the amino acid analyser. Alternatively it could be that there is the loss of a small protein component from the fractions during the dialysis steps. It should be noted that an accurate estimation of cysteine was not achieved. This possibly can be explained by incomplete alkylation of all the cysteine residues in the fractions. The reduction and alkylation was

carried out in the presence of 8M urea-DTT solutions, however, as can be seen (Table XVI) complete solubilization of the fractions does not occur in this solvent.

The Sephadex G-200 profiles for reduced and alkylated A and G in 1% SDS were similar to that obtained by Wolff et al (144) for SGP from rat skin dissolved in urea. SDS electrophoretic patterns obtained by these workers for urea soluble and crosslinked structural glycoproteins from rabbit skin showed up to 15 bands from which it was concluded that these materials were heterogeneous. The observation was made, however, that some of the heterogeneity "might be the result of different aggregation states of a single polypeptide chain". The SDS electrophoretic system used by these workers was the original procedure of Shapiro et al (6) which is a continuous electrophoresis system in 0.1M phosphate buffer (pH 7.1) with 0.1% SDS. The results presented in this thesis show that a discontinuous electrophoresis system in Tris buffer with 0.1% SDS (Procedure D) produces a much simpler electrophoretic profile. The work presented in this thesis indicates aggregation of these glycoproteins is a primary reason for the observed heterogeneity. Aggregation of glycoproteins in SDS is not a unique observation (181, 182, 183, 184, 185) and it has been proposed as a general property of water insoluble or membrane glycoproteins (182, 185, 186).

Although aggregation of A and G was clearly demonstrated by SDS gel electrophoresis, it could not be demonstrated by gel chromatography alone.

Concentration and rechromatography of peaks fractionated on Sephadex G-200 did not show redistribution of protein. However, the presence of considerable amounts of solubilized Sephadex dextran in these peak pools may have altered the interaction of the protein and thus invalidate the conclusions. No evidence of multiple molecular forms could be demonstrated in the analytical ultracentrifuge which indicated that both fractions consist of a single, though probably polydisperse, species. Compatibility between ultracentrifugal and column chromatographic data is not axiomatic. The Sephadex G-200 columns were run in 1% SDS, whereas the ultracentrifugation studies were carried out in 0.1% SDS. Moreover, Hardingham and Muir (13) have shown that hyaluronic acid-proteoglycan interactions do not yield aggregates which can be observed in the ultracentrifuge, although mixtures of the two have higher viscosities than proteoglycan solutions alone and give excluded peaks on Sepharose 2B columns which are not present in the absence of hyaluronic acid.

The rather erratic relationship between sedimentation velocity and concentration has no explanation at this time. Tanford's detailed studies on the molecular characterization of proteins in SDS solutions (176) have shown that the sedimentation velocity of the protein-SDS complex is extremely sensitive to protein concentration. When one considers that Fractions A and G are glycoprotein and it is known that the presence of carbohydrate can affect both detergent binding and the hydrodynamic behaviour of the detergent-protein complex (185) such results become less unexpected. No attempts were made to assign a molecular weight to the isolated fractions on the basis of the gel chromatographic or electrophoretic data as this demands firstly, that the amount of SDS bound per gram of protein is the same for the glycoprotein as for standard water soluble

proteins and secondly, that the relationship between the hydrodynamic size of the glycoprotein and its molecular weight must be identical to that relationship for water soluble proteins.

The similarities between reduced and alkylated A and G on SDS gel chromatography and electrophoresis are remarkable. This, together with the similarity in amino acid composition of SDS-treated A and G (Table XIX), suggests the possibility that some small protein component was lost during the dialysis of these solubilized preparations, leaving a common unique protein in both fractions. Further work is required to establish the validity of this suggestion. The main conclusions from the data relating to the non-dialysable solubilized A and G are that they behave as homogeneous materials in SDS. Upon electrophoresis on 8 or 10% polyacrylamide and in the ultracentrifuge they show evidence that each is a single but polydisperse species. Aggregated forms of A and G were found on 12% polyacrylamide and Sephadex G-200.

Electron microscopy of A and G and their relationship to histologically observed structures.

The appearance of Fraction G in electron micrography (Figures 37a-d) can be accounted for very largely by the presence of fine filaments, either singly or aggregated into large groups which occasionally have the form of an outline of the original collagen fiber (Figure 34c, d). In contrast, the usual size of the particles seen in A is smaller, though some

of the filaments seen in G may be present (Figure 36a, b). That these electron microscope observations are not artefactual is indicated by two things. Firstly, the appearance of Fractions A and G is very similar to the material surrounding the collagen fibers of Fraction 1. After treatment with alcian blue-PTA, filaments of 60-100A° diameter and 200-1000A° length are seen in regular array along most of the recognizable collagen fibers (Figure 34c,d). Many of the collagen fibers also have fuzzy portions (Figure 34c, d) where the appearance of the material around the fibers is similar to that of Fraction A (Figure 36a, b). Fine filaments do not show up on collagen fibers treated with PTA alone (Figure 34a, b). When Fraction 1 was treated with lead, or ruthenium red, filaments were occasionally visible, but, when present, they were much fainter than with alcian-PTA, (Figure 35a, b, c). However, on all parts of the collagen fibers there was a regular array of dark spots or protuberances which were spaced along the fibers with the same periodicity as the filaments and the 700A° collagen bands (Figure 35a, b, c). It seems possible that these dark areas are filaments which have coiled into globules, but whatever their form it is clear that they stain with lead of ruthenium red more strongly than the main part of the collagen fibers. It therefore seems likely that these localized areas consist of acidic material such as that in Fractions A and G. Secondly, the appearance of the material surrounding the collagen fibers of Fraction 1 is very similar to that which has been observed by other workers in sections of intact tissues. The attachment of fine orthogonal filaments to the 700A° cross-bands of collagen, as seen in Figures 34c and d, was postulated by Meyer (187) and observed by Smith and Frame (188) in cornea using a bismuth nitrate stain and in articular cartilage, synovium

and cornea by Myers, Highton and Rayns (94, 95) using ruthenium red. In addition, the latter group found fuzzy material around portions of the collagen fibers. The filaments were not isolated, but it was proposed that they were aggregates of glycoproteins and proteoglycans (94, 95). There are several reasons why it is concluded that the filaments observed in the present work are mainly glycoprotein. Although, on the basis of the glucuronate and galactosamine content (Table IX), and paper electrophoresis (Figure 11), it appears that there is a chondroitin sulfate component in both Fractions A and G; the content in Fraction G (1-5%) is so low that is cannot account for all the filaments seen in electron micrographs of this fraction. In addition, the A and G particles are much smaller than the proteoglycans observed by Rosenberg et al (75). The amino acid composition of Fractions A and G (Table XVII) is quite different from that found for the core protein of cartilage proteoglycan (12) which, for example, has a much higher content of serine and proline and a much lower content of aspartic acid and lysine. In this connection it may be noted that alcian blue will react with acid groups other than those in the acid GAGs (157) and that both Fractions A and G have a high content of acidic amino acids.

Electron micrographs taken after addition of soluble proteoglycan to Fraction 1 show the presence (Figure 35d) of a larger number of interconnecting fine filaments than are found in the insoluble collagenous fractions alone. This suggests that proteoglycan molecules may link up with the orthogonal glycoprotein filaments on the collagen fibers. This suggestion is also in agreement with the finding that short, lateral filaments remain attached

to collagen fibers after guanidine hydrochloride or hyaluronidase digestion of tissue sections has removed the main portion of the links between the collagen fibers (95). The electron microscopic evidence indicates that these attachments to collagen can be separated by the procedure described in this thesis and their composition indicates that they are mainly glycoprotein.

From a chemical point of view, the non-destructive salt extraction and the digestion with highly purified collagenase would not be expected to cleave covalent bonds in Fractions A or G (2,7), therefore it is proposed that these fractions are native molecules.

The only other situations where structures with similar amino acid compositions have been visualized are the 75-100A° diameter pathologic amyloid fibrils observed in a number of different tissues (189, 191, 216), and the 110A° diameter microfibrils present in developing elastic tissue (20). The fibrillar glycoprotein Fraction G resembles these two other acidic fibrillar proteins in amino acid composition and solubility (20, 189, 190). The diameters of microfibrils and amyloid fibrils are considerably larger than those of the G filaments seen in this thesis. A very recent paper (10) indicates that microfibrils are tubular. These tubules look like bundles of filaments with the diameter of those in Fraction G.

Interactions between A,G and other components of cartilage.

Interactions between extracellular matrix macromolecular components are of importance since they may determine the organization and structural integrity of the cartilage. The insoluble glycoprotein Fractions A and G contain residual amounts of collagen and chondroitin sulfate proteoglycan (Table IX, Figure 11). The question arises as to the nature of the

association of these macromolecular components with the insoluble glycoprotein fractions.

It could be that:

(i) All of the components are associated only because they are all insoluble. However, to date no insoluble acid GAG or proteoglycan has been separated and isolated from any source.

(ii) There is a high degree of physical entanglement of the glycoprotein, residual proteoglycan and fragments of collagen. There are several pieces of evidence which are strongly against this as an explanation for the insolubility of the non-collagen components. A and G exhaustively digested with collagenase remain insoluble. The trace levels of hydroxyproline (0.08-0.1% in Table XI) in both fractions concurrent with the presence of 0.9-2.4% glucuronate (Table XI) reasons against physical entanglement with undigested insoluble collagen as an explanation for the insolubility of Fractions A , G and associated proteoglycan. In addition, electron micrographs of A and G (Figure 36a, 37a) after collagenase digestion show relatively few recognisable collagen fibers in accordance with low levels of hydroxyproline.

(iii) There are covalent bonds between all of these components.

(iv) There is a non-covalent binding of the type found in lectin-glycoprotein (114), glycoprotein-proteoglycan (77,12, 115) and collagen-glycoprotein (17) interactions.

The solubility properties of A and G shed some light on these last two points. Extraction of A and G with guanidine HCl or sodium acetate (Tables XXV, XXVI) indicate that at least one-half of the residual chondroitin sulfate proteoglycan is not held in covalent linkages as these solvents do not cleave covalent bonds. The status of the residual non-extractable glucuronic acid in A and G is unresolved. The work of Herbage et al (111)

as an explanation for the insolubility of proteoglycan would seem not to apply to Fractions A and G. In this study it was shown that upon destruction of the collagen helical structure by the use of dissociative solvents (92, 111), undefined new interactions between denatured collagen and proteoglycan are created which results in the irreversible insolubilization of the latter. In A and G the proteoglycan remains insoluble even when essentially all of the collagen has been removed (Table XI). As well, treatment of A and G with 8M urea-DTT solutions (Table XVI) solubilized most of the non-collagen protein and glucuronate as opposed to the action of 8M urea alone. Both solvents leave the collagenous component insoluble. This indicates a non-covalent attachment between collagen and the non-collagen components. If the glucuronic acid containing material were covalently linked with collagen, it would have remained insoluble.

In summary, the insolubility of residual proteoglycan and of glycoprotein in Fraction A and G is not due to physical entanglement with insoluble collagen nor to covalent linkages between these components and insoluble collagen. The presence of unextractable proteoglycan leaves the possibility of a covalent linkage of a portion of the proteoglycan to glycoprotein an open question. Evidence for a covalent linkage between chondroitin sulfate and a glycoprotein obtained from EDTA extracts of bone has been found by Herring (27), however, none has been documented in mammalian cartilage.

The physiological role of structural glycoproteins is at present not understood, but some of the possible functions of these substances have been noted in Chapter 1. The tenacious association of a portion of the proteoglycan to Fractions A and G suggests that these glycoproteins may be the anchoring material for that portion of cartilage proteoglycan which is

not easily extracted and which may have a distinct function in calcification (150).

Conclusions

Two insoluble non-collagenous glycoprotein fractions (A and G) have been separated from puppy rib cartilage, following extraction of most of the proteoglycan and digestion of the insoluble residue with purified collagenase.

The solubility properties of A and G are similar to those of structural glycoproteins (SGP) extracted from other tissues. The amino acid compositions of both fall within the range found for SGP by other workers.

After reduction, alkylation and extraction with sodium dodecyl sulfate most of each protein is solubilized. Gel electrophoresis of solubilized A or G shows the presence of either one or two bands and gel chromatography shows both high and low molecular weight peaks. The production of a low molecular weight electrophoresis band from the high molecular weight Sepahdex fraction indicates that there is aggregation and disaggregation of subunits in sodium dodecylsulfate. Analytical ultracentrifugation of solubilized A and G showed that both fractions sediment as a single symmetrical peak, but, with different S values. No evidence of aggregation was demonstrable using this technique.

Both A and G are high in aspartate plus glutamate and have a low hydroxyproline content. The insoluble A and G both contain hexose, uronic acid, galactosamine, glucosamine and a small amount of sialic acid, but they differ in their contents of hexose and six amino acids. Both fractions contain bound chondroitin sulfate. They both form single bands in CsCl gradients but they differ in density.

Biochemical and electron microscopic evidence shows that both insoluble fractions are in close association with collagen. Electron microscopy shows that Fraction G is filamentous whereas Fraction A appears amorphous. Material with the same appearance was found to occur on the surface of collagen fibers in the undigested cartilage residue and the diameter and staining properties of the G filaments are very similar to those of filaments found by other workers in intact cartilage sections.

TABLE I

COMPOSITION AND DISTRIBUTION OF SOME ACID GAGs

POLYSACCHARIDE	MOLECULAR WEIGHT	CONSTITUENTS	BIOLOGICAL SOURCE
Hyaluronic acid	4×10^5 $2 - 10 \times 10^6$	Glucuronic acid; N-Ac glucosamine	Vitreous body Synovial fluid
Chondroitin		Glucuronic acid; N-Ac galactosamine	Cornea
Chondroitin - 4 -sulfate	$1.5 - 2 \times 10^4$	Glucuronic acid; N-Ac galactosamine 4-sulfate	Costal cartilage Ox nasal septa
Chondroitin - 6 -sulfate	$1.5 - 2 \times 10^4$	Glucuronic acid; N-Ac galactosamine 6-sulfate	Cartilage
Dermatan sulfate	2.7×10^4	Iduronic acid; N-Ac galactosamine 4-sulfate	Skin
Keratan sulfate	$1 - 2 \times 10^4$	Galactose; galactose 6-sulfate; N-Ac glucosamine 6-sulfate	Cornea
Heparan sulfate		Glucuronic acid 2-sulfate; glucosamine 6-sulfate N-Ac or N sulfate	Aorta
Heparin	1×10^4	Glucuronic acid 2-sulfate; glucosamine 6-sulfate, N sulfate	Lung

TABLE II

EXTRACTION OF PROTEOGLYCAN FROM PUPPY RIB CARTILAGE

	Pooled $CaCl_2$ Extracts	Pooled Water Extracts	Insoluble Residue (Fraction 1)	Total
Recovery of Glucuronolactone (mg/gm fresh tissue)	6.63	3.86	6.58 (6.16)*	17.07
Distribution (%)	38.9	22.6	38.5	100

* when 4M Guanidine HCl was used to extract the ribs

TABLE III

EFFECT OF PARTIALLY PURIFIED COLLAGENASE ON FRACTION 1 ACCORDING TO PROCEDURE 1

Time	µmoles solubilized / 20 mg Fraction 1	
	Glucuronic Acid *	Hydroxyproline *
1h	3.7	9.9
2h	4.8	10.9
3h	5.8	12.6
4h	6.1	13.1
23h	-	13.4

* The values are cumulative and represent the total amount of each component which has been solubilized up to the given time.

TABLE IV

ANALYSIS OF FRACTIONS ISOLATED AFTER DIGESTION OF FRACTION 1 WITH PARTIALLY PURIFIED COLLAGENASE ACCORDING TO PROCEDURE 1

	% Composition					
	Hydroxy-proline	Collagen†	Glucuronic Acid	Sialic Acid	Total Lowry Protein	Non-Collagenous Protein *
Fr. 1	8.4	60.1	4.3	0.20	65.9	18
D_1	6.3	45	1.2	0.16	58.5	30.5
D_2	0.4	2.6	19.3	0.50	21.6	20.0
D_3	0.3	2.1	2.0	0.66	86.0	84.7

D_1 Non-dialysable material solubilized by the digestion (MW 10,000-50,000)

D_2 Non-dialysable material solubilized by the digestion (MW >50,000)

D_3 Insoluble residue after digestion.

† Obtained from the hydroxyproline value using the factor 7.15.

* Total Lowry protein corrected for the collagen content in each sample. This is done by subtracting the Lowry protein equivalents of an amount of soluble calf skin collagen which is equal to the collagen content of the analysed fraction. Inherent is the assumption that equal amounts of calf skin collagen and the collagen in the samples produce the same number of equivalents of the bovine serum albumin standard.

TABLE V

RECOVERY OF COMPONENTS OF NON-DIALYSABLE FRACTIONS ISOLATED AFTER DIGESTION OF FRACTION 1 WITH PARTIALLY PURIFIED COLLAGENASE ACCORDING TO PROCEDURE 1

	Recovery (%) of the amount in Fraction 1				
	Hydroxy-proline	Glucuronic Acid	Sialic Acid	Total Lowry Protein	Non-collagen Lowry Protein *
D_1	10.2	3.7	10.0	12.1	23.1
D_2	1.0	91.9	52.5	6.8	22.8
D_3	0.2	2.6	17.5	7.3	26.3
Total	11.4	98.2	80.0	26.2	72.2

* Total Lowry protein corrected for collagen.

TABLE VI

EFFECT OF PURIFIED COLLAGENASE ON FRACTION 1 ACCORDING TO PROCEDURE 2

Time	µmoles solubilized / 20 mg Fraction 1			
	Glucuronic Acid *		Hydroxyproline *	
0.5h	1.18		5.16	
1h	1.43		4.50	
2h	1.63		5.46	
3h	1.97		6.49	
4h	2.18		6.07	
5h	2.35		6.89	
23h	3.36	75.8†	8.08	63.1†

* The values are cumulative and represent the total amount of each component which has been solubilized up to the given time.

† Percentage of this component present in Fraction 1 which is solubilized up to this time.

TABLE VII

EFFECT OF PURIFIED COLLAGENASE ON FRACTION 1

ACCORDING TO PROCEDURE 3

Time	µmoles solubilized / 20 mg Fraction 1	
	Glucuronic Acid *	Hydroxyproline *
0.5h	1.13	2.18
1.0h	1.36	4.39
1.5h	1.46	5.62
2.0h	1.56	6.87

* The values are cumulative and represent the amount of each component which has been solubilized up to the given time.

TABLE VIII

ANALYSIS OF INSOLUBLE RESIDUE AFTER DIGESTION OF FRACTION 1 WITH PURIFIED COLLAGENASE ACCORDING TO PROCEDURE 3

	% Composition		
	Hydroxy-proline	Collagen *	Glucuronic Acid
Fraction 1	8.4	37	5.7
Insoluble Residue after collagenase	2.7	19.2	4.9

* Obtained from the hydroxyproline value using the factor 7.15.

TABLE IX

RECOVERY AND COMPOSITION OF INSOLUBLE CARTILAGE FRACTIONS OBTAINED BY PROCEDURES 4 AND 5.

Treatment	Guanidine or $CaCl_2$ extraction	Collagenase digestion		
		Low speed pellet	High speed pellet	
Insoluble residue (washed and freeze dried)	Fraction 1	Fraction 2	Upper layer Fraction A	Lower layer Fraction G
Recovery (mg/g fresh cartilage)	150-300	10-20	2-4	6-8
Percentage composition				
Hydroxyproline	8.4 ± 0.6	5.8 ± 0.3	0.8 ± 0.2	0.7 ± 0.1
Collagen*	60.1	41.5	5.7	5.0
Glucuronate	4.3 ± 0.3	4.6 ± 0.6	3.0 ± 0.6	1.7 ± 0.2
Hexose	6.4 ± 0.2	6.0 ± 0.7	5.1 ± 0.4	7.1 ± 0.8
Non-collagenous Lowry protein†	18 ± 2	28 ± 3	51 ± 2	56 ± 3
Glucosamine			1.4	0.5
Galactosamine			4.8	1.5
Sialic acid	0.2	0.2	0.5	0.4

The single figures are means of two estimates only. Other figures are means ± S.E. for at least 8 estimates on 4 separately prepared samples.

* Obtained from the hydroxyproline mean using the factor of 7.15.

† Total Lowry protein corrected for collagen.

TABLE X

ANALYSIS OF FRACTIONS A AND G SEPARATED IN BUFFER *

	% Composition	
	Hydroxyproline	Collagen †
Fraction A	4.6	32.9
Fraction G	3.9	27.9

† Obtained from the hydroxyproline value using the factor 7.15.

* 0.05 M TRIS-HCl, 0.5 M $CaCl_2$, 0.02% NaN_3, pH 7.4.

TABLE XI

ANALYSIS OF FRACTIONS A AND G AFTER EXHAUSTIVE COLLAGENASE DIGESTION

	Fraction A	Fraction G
Hydroxyproline	0.1	0.08
Collagen†	0.7	0.6
Glucuronate	2.4	0.9
Hexose	6.3	3.9
Total Lowry Protein	71.1	56.2

† Obtained from the hydroxyproline value using the factor 7.15.

TABLE XII

SOLUBILIZATION OF COMPONENTS OF FRACTION A

IN 0.02N NaOH FOR 19 h AT 23°

	% INSOLUBLE	% SOLUBLE	% RECOVERY
Hydroxyproline	85	0	85
Total Lowry Protein	68	50.5	119
Glucuronic Acid	0	117	117
Hexose	51	56	106
†Non-Collagenous Lowry Protein	62	50.5	112.5

† Total Lowry protein corrected for collagen

TABLE XIII

SOLUBILIZATION OF COMPONENTS OF FRACTION G IN 0.02N NaOH FOR 19 h AT 23°

	% INSOLUBLE	% SOLUBLE	% RECOVERY
Hydroxyproline	77	0	77
Total Lowry Protein	59	51	110
Glucuronic Acid	25	77	102
Hexose	92	26	118
†Non-Collagenous Lowry Protein	58	51	109

† Total Lowry protein corrected for collagen

TABLE XIV

SOLUBILIZATION OF COMPONENTS OF FRACTION A

IN 8M UREA AT 4°

	% OF COMPONENT UNDISSOLVED	
	AFTER 19 h	AFTER 48 h
Hydroxyproline	86	76
Total Lowry Protein	91	87
Glucuronic Acid	79	83
Hexose	91	88
†Non-Collagenous Lowry Protein	87	89

† Total Lowry protein corrected for collagen

TABLE XV

SOLUBILIZATION OF COMPONENTS OF FRACTION G

IN 8M UREA AT 4°

	% OF COMPONENT UNDISSOLVED	
	AFTER 19 h	AFTER 48 h
Hydroxyproline	68	76
Total Lowry Protein	97	86
Glucuronic Acid	67	84
Hexose	93	99
†Non-Collagenous Lowry Protein	100	90

† Total Lowry protein corrected for collagen

TABLE XVI

SOLUBILIZATION OF COMPONENTS OF FRACTIONS A AND G IN 8M UREA WITH 0.01M DITHIOTHREITOL

	% OF COMPONENT UNDISSOLVED AFTER 48 h AT 4°	
	FRACTION A	FRACTION G
Hydroxyproline	81	78
Total Lowry Protein	20	31
Glucuronic Acid	17	33
Hexose	46	82
†Non-Collagenous Lowry Protein	17	29

† Total Lowry protein corrected for collagen

TABLE XVII

AMINO ACID COMPOSITION OF PUPPY RIB CARTILAGE GLYCOPROTEINS

RESIDUES PER 1000 RESIDUES NON-COLLAGENOUS PROTEIN *

AMINO ACID	MEANS ± S.E. FOR 4 SAMPLES, SEPARATELY HYDROLYSED			Single sample of link glycoprotein
	FRACTION A	FRACTION G	P FOR 'T' TEST	
LYS	50 ± 4	68 ± 1	<0.01	40
HIS	21 ± 2	22 ± 1		21
ARG	54 ± 2	54 ± 2		57
ASP	123 ± 4	104 ± 1	<0.01	117
THR	52 ± 2	50 ± 1		56
SER	56 ± 4	51 ± 2		54
GLU	116 ± 2	124 ± 2	<0.05	111
PRO	63 ± 1	54 ± 1	<0.01	69
GLY	98 ± 14	86 ± 2		93
ALA	67 ± 3	76 ± 2	<0.02	80
CYS/2	25 ± 3	16 ± 4		14
VAL	68 ± 3	69 ± 1		67
MET	10 ± 3	14 ± 5		6
ILE	44 ± 2	51 ± 1	<0.02	37
LEU	94 ± 3	96 ± 2		87
TYR	26 ± 6	26 ± 4		51
PHE	36 ± 1	38 ± 1		41
ASP/GLU	1.09± 0.04	0.84± 0.01	<0.01	1.05

* Calculated by correcting the total amino acid analyses for the various collagen amino acids, as obtained from the hydroxyproline content and the amino acid composition of bone collagen. (217)

TABLE XVIII

RECOVERY OF FRACTIONS A AND G UPON REDUCTION AND ALKYLATION FOLLOWING METHOD 1

	TOTAL LOWRY PROTEIN (μg)		
	Before Reduction and Alkylation	After Reduction and Alkylation	% Recovery
Fraction A	646.6	561.5	86.8
Fraction G	645.2	608.5	94.3

TABLE XIX

AMINO ACID COMPOSITION OF REDUCED & ALKYLATED A & G

RESIDUES PER 1000 RESIDUES OF NON-COLLAGENOUS PROTEIN *		
AMINO ACID	SINGLE SAMPLE A	SINGLE SAMPLE G
Lys	51	46
His	21	18
Arg	53	54
S-Cmc †	17	29
Asp	124	111
Thr	55	54
Ser	69	64
Glu	112	110
Pro	66	61
Gly	102	97
Ala	70	82
Val	63	63
Met	2	10
Isoleu	41	41
Leu	86	91
Tyr	15	25
Phe	54	44
Asp/Glu	1.10	1.01

* Corrected for the low collagen amino acid content as described in Table XVII.

† The S-Cmc values represent the sum of the S-carboxymethylcysteine and cysteine residues in the incompletely alkylated samples.

TABLE XX

SOLUBILIZATION OF REDUCED AND ALKYLATED (^{14}C-IODOACETIC ACID) FRACTION A AT 23° FOR 24 h

FRACTION A IN	ACTIVITY IN SUPERNATANT (dpm/mg Fraction A)	% COUNTS DISSOLVED
Buffer	5805	44.1
Buffer + 0.1% SDS	5301	40.2
Buffer + 1.0% SDS	13495	102.0†
Buffer + 5% SDS	13660	103.6†
Buffer + 8M Urea	12192	92.3†

†means of 2 trials

Buffer 0.1M phosphate, pH 7.0, 0.02% NaN_3

S.A. Fraction A = 13170 dpm/mg dry weight

TABLE XXI

SOLUBILIZATION OF REDUCED AND ALKYLATED (^{14}C-IODOACETIC ACID) FRACTION G AT 23° FOR 24 h

FRACTION G IN	ACTIVITY IN SUPERNATANT (dpm/mg Fraction G)	% COUNTS DISSOLVED
Buffer	7848	62.1
Buffer + 0.1% SDS	11532	91.3
Buffer + 1.0% SDS	11424	90.9 †
Buffer + 5.0% SDS	11847	92.6 †
Buffer + 8M Urea	10717	84.7 †

† means of 2 trials

Buffer 0.1M phosphate, pH 7.0, 0.02% NaN_3

S.A. Fraction G = 12630 dpm/mg dry weight

TABLE XXII

RECOVERY OF REDUCED AND ALKYLATED FRACTIONS A AND G UPON SOLUBILIZATION IN 0.1M PHOSPHATE BUFFER, 0.02% NaN_3 5.0% SDS, pH 7.0 at 37° FOR 48 h

LOWRY PROTEIN µg/mg DRY WEIGHT OF REDUCED AND ALKYLATED FRACTIONS

	BEFORE SDS TREATMENT	SUPERNATANT AFTER SDS	PELLET AFTER SDS	% RECOVERY
Fraction A	612	623.1	8.9	103.2
Fraction G	632	523.1	24.4	86.6

TABLE XXIII

COMPONENTS OF FRACTIONS A AND G AFTER TREATMENT BY REDUCTION, ALKYLATION AND EXTRACTION WITH 0.1M PHOSPHATE BUFFER, 0.02% NaN_3, 5.0% SDS, pH 7.0 at 37° for 48 h

	µg per mg assayed (Lowry) protein				
	Glycine	Aspartate	Glutamate	Hydroxyproline	Hexose
Fraction A					
Before Treatment	17.2	34.0	34.3	4.0	128.0
Solubilized by Treatment	17.2	39.2	38.0	1.1	195
Insoluble After Treatment †	63.1	19.2	38.5	-	-
Fraction G					
Before Treatment	18.7	32.8	35.9	6.5	66.1
Solubilized by Treatment	15.7	34.9	37.3	2.6	135
Insoluble After Treatment †	68.4	13.8	29.5	-	-

† The amounts of material insoluble after treatment were too small to complete the hydroxyproline and hexose assays.

TABLE XXIV

AMINO ACID COMPOSITION OF SDS SOLUBLE, REDUCED & ALKYLATED A AND G

	RESIDUES PER 1000 RESIDUES	
AMINO ACID	SINGLE SAMPLE A	SINGLE SAMPLE G
Lys	46	48
His	18	17
Arg	54	50
S-Cmc †	5	13
Asp	131	118
Thr	55	54
Ser	76	67
Glu	115	114
Gly	102	94
Ala	73	77
Val	63	57
Met	-	-
Isoleu	36	36
Leu	85	78
Tyr	28	29
Phe	54	43
Asp/Glu	1.14	1.04

† The S-Cmc values represent the sum of the S-carboxymethylcysteine and cysteine residues in the incompletely alkylated samples.

TABLE XXV

EXTRACTION OF GLYCOSAMINOGLYCAN URONIC ACID FROM A AND G BY 4.0M GUANIDINE HCl

	GLUCURONIC ACID (µg)				
	Before Extraction	After Extraction		% Recovery	% Extracted
		Supernatant	Pellet		
Fraction A	39.4	22.4	17.3	100.8	56.8
Fraction G	21.2	12.0	8.4	96.4	56.6

TABLE XXVI

EXTRACTION OF GLYCOSAMINOGLYCAN URONIC ACID

FROM A AND G BY 0.5M SODIUM ACETATE

	GLUCURONIC ACID (µg)			
	Before Extraction	After Extraction		% Extracted
		Supernatant	Pellet	
Fraction A	101.8	31.5	N.D.	30.6
Fraction G	70.1	24.5	N.D.	34.9

N.D. - not determined

Figure 1

Procedure 4

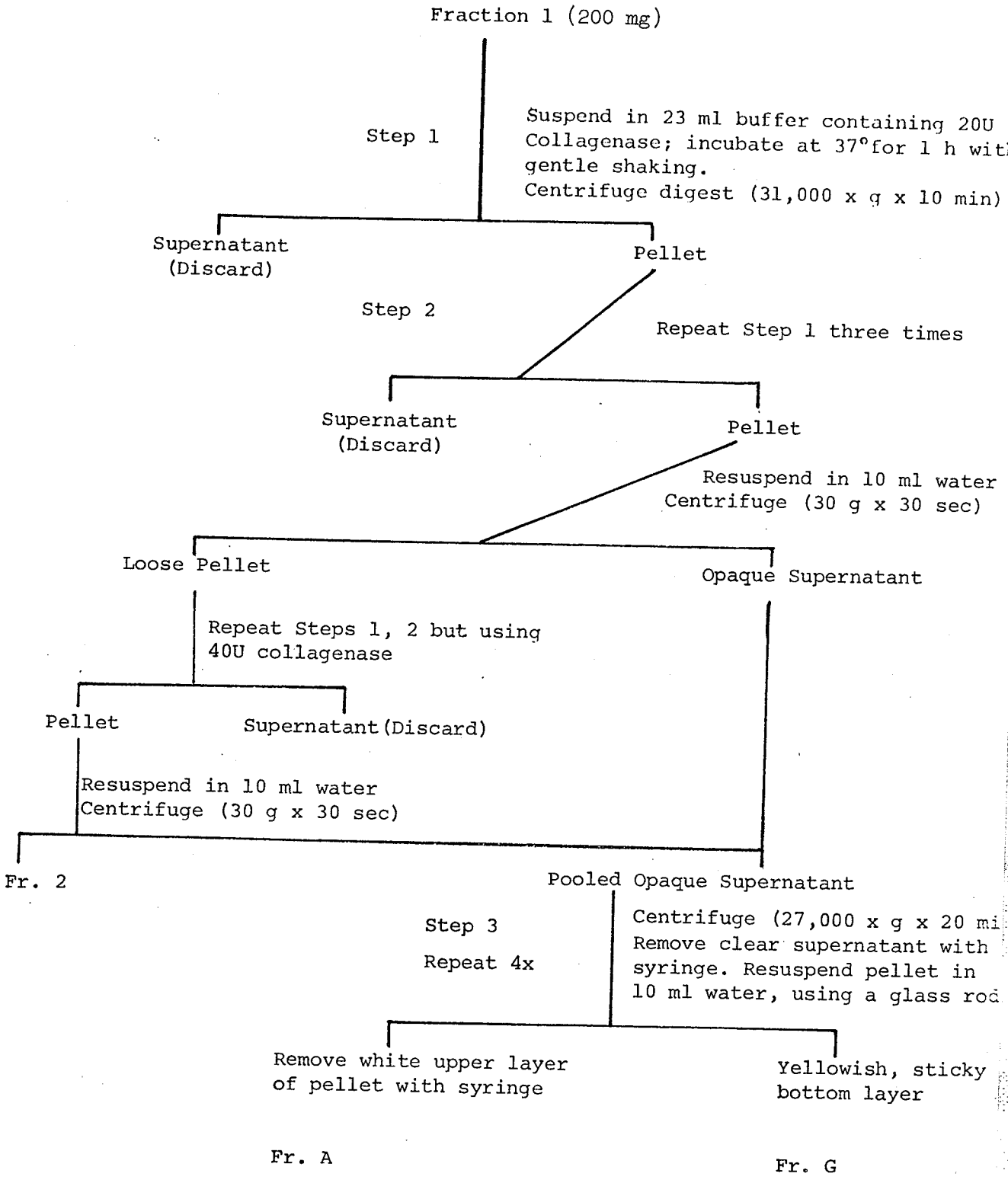
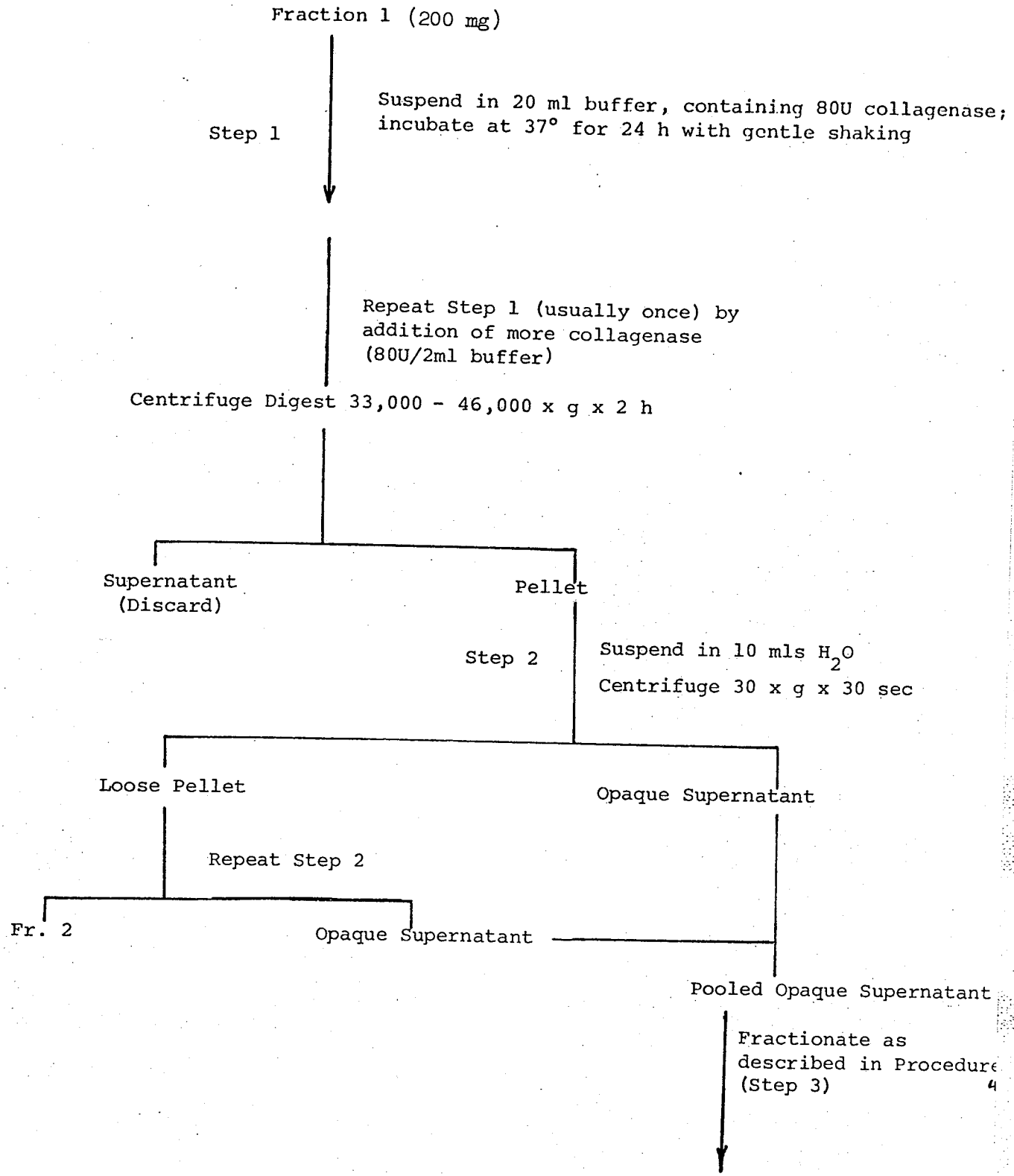


Figure 2

Procedure 5



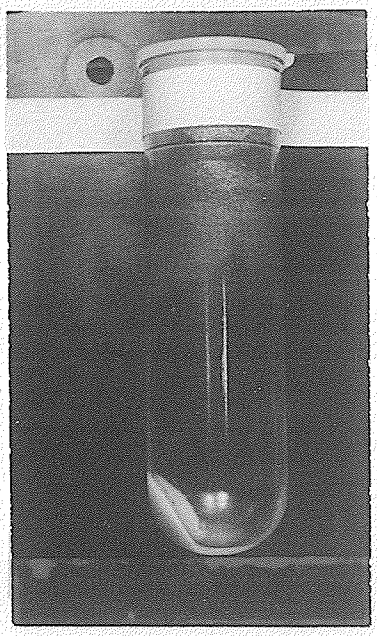


Figure 3

Opaque Supernatant from Procedure 5 before water washing. Spun at 27,000 - 35,000 x g x 20 min.

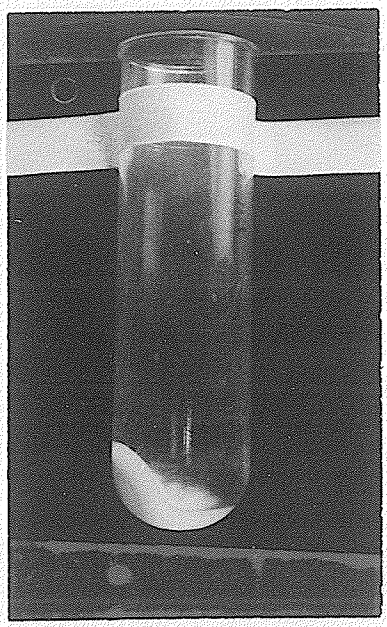


Figure 4

Opaque Supernatant from Procedure 5 after water washing. Spun at 27,000 - 35,000 x g x 20 min.

Fraction A - Upper white layer

Fraction G - Lower yellow layer

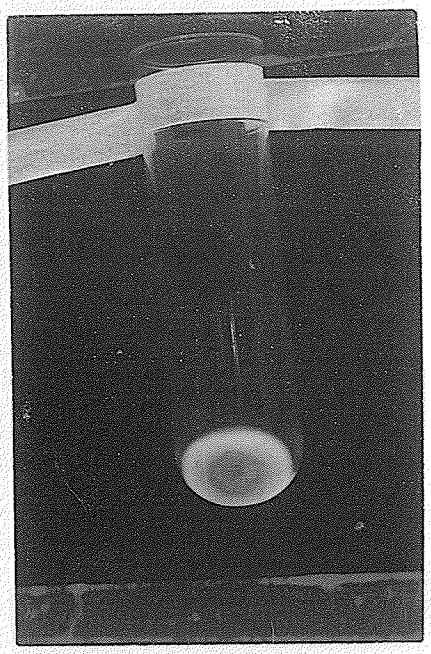


Figure 5

Opaque Supernatant from Procedure 5 after water washing. Spun at 27,000 - 35,000 x g x 20 min.

Fraction A - Upper white layer

Fraction G - Lower yellow layer

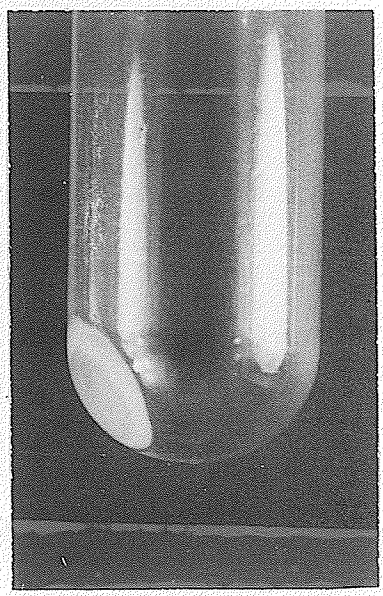


Figure 6

Insoluble layers (A and G) in the collagenase digestion buffer. Spun at 27,000 - 35,000 x g x 20 min.

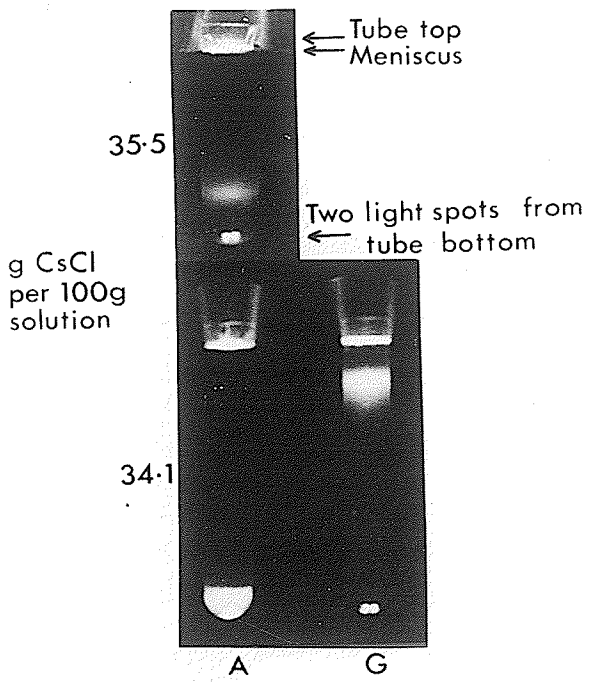


Figure 7

Bands formed by centrifuging Fractions A or G in CsCl solutions for $2.1 \times 10^6 \times g_{av} \times h$. The two spots at the bottom of each tube are due to reflections from the lamps during photography.

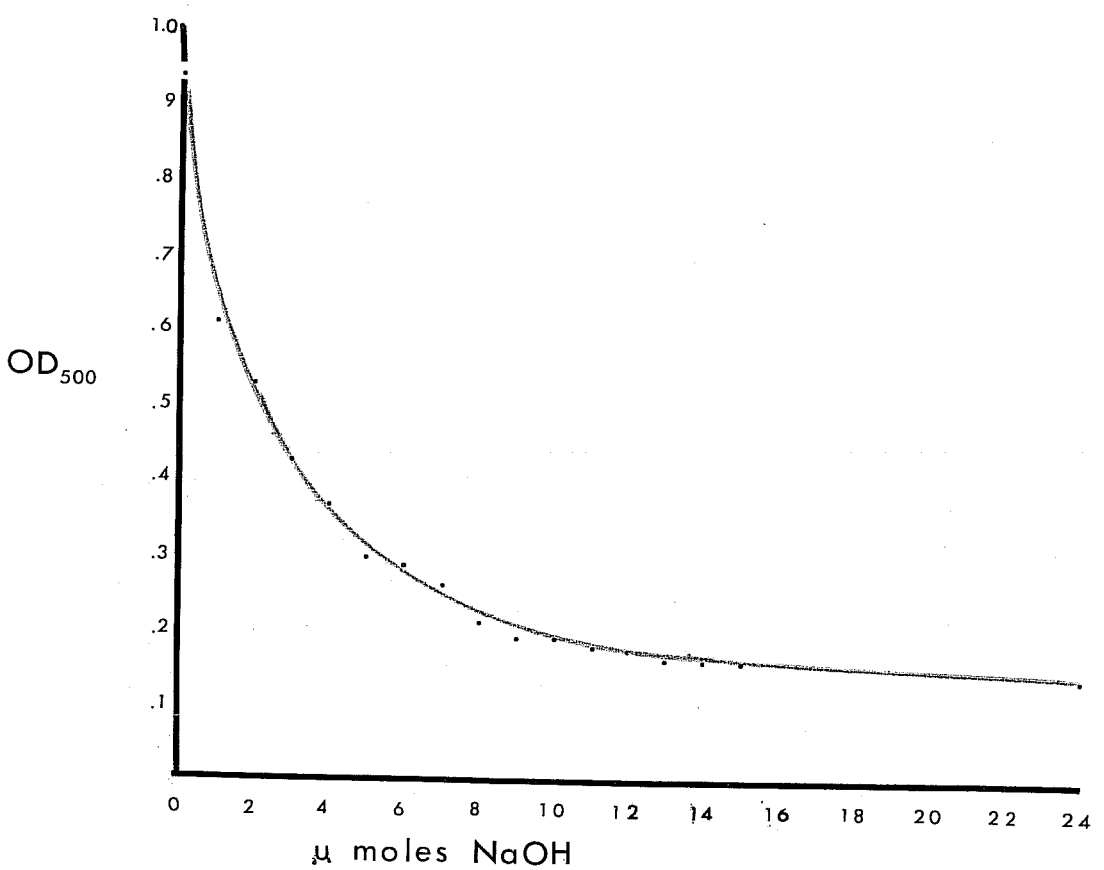


Figure 8

Solubilization of Fraction G in NaOH

After each addition of NaOH (2 μmoles) the suspension was heated at 100° for 2 min. Upon cooling the OD_{500} was read against a water blank.

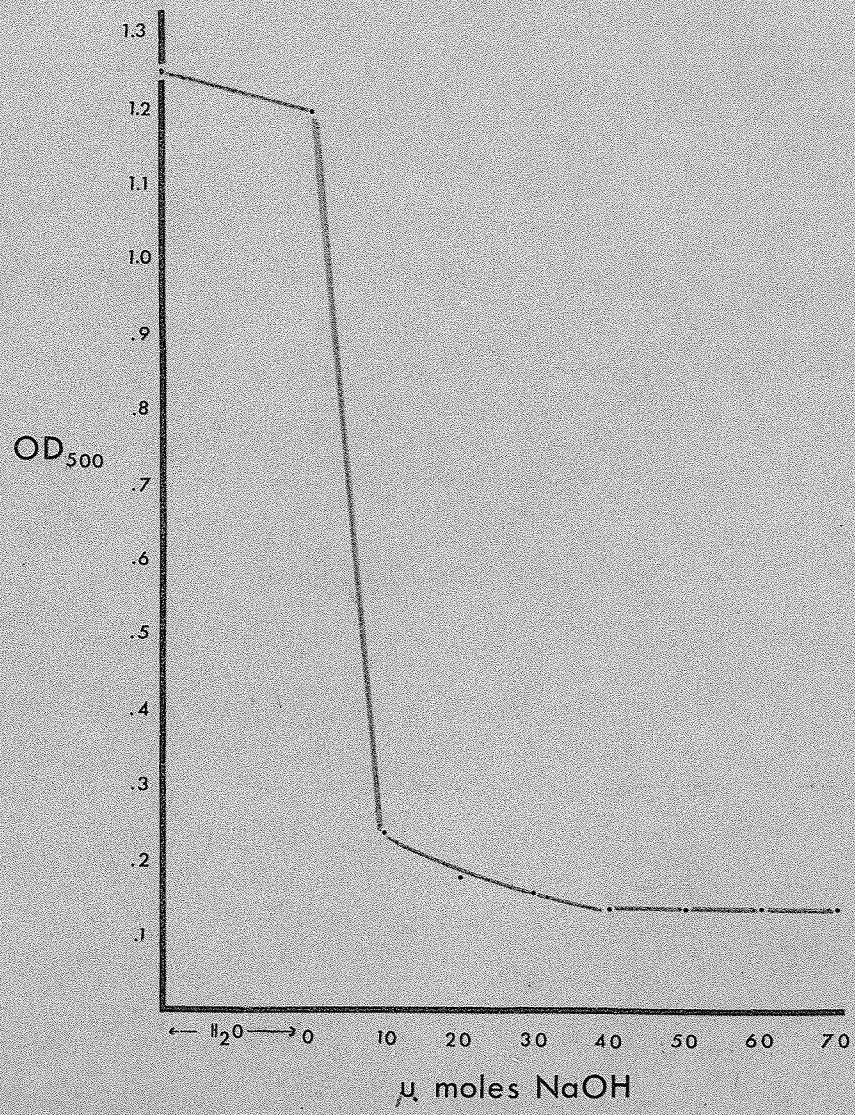


Figure 9

Solubilization of Fraction G in NaOH

After each addition of NaOH (10 μmoles) the suspension was heated at 100° for 2 min. Upon cooling the OD_{500} was read against a water blank.

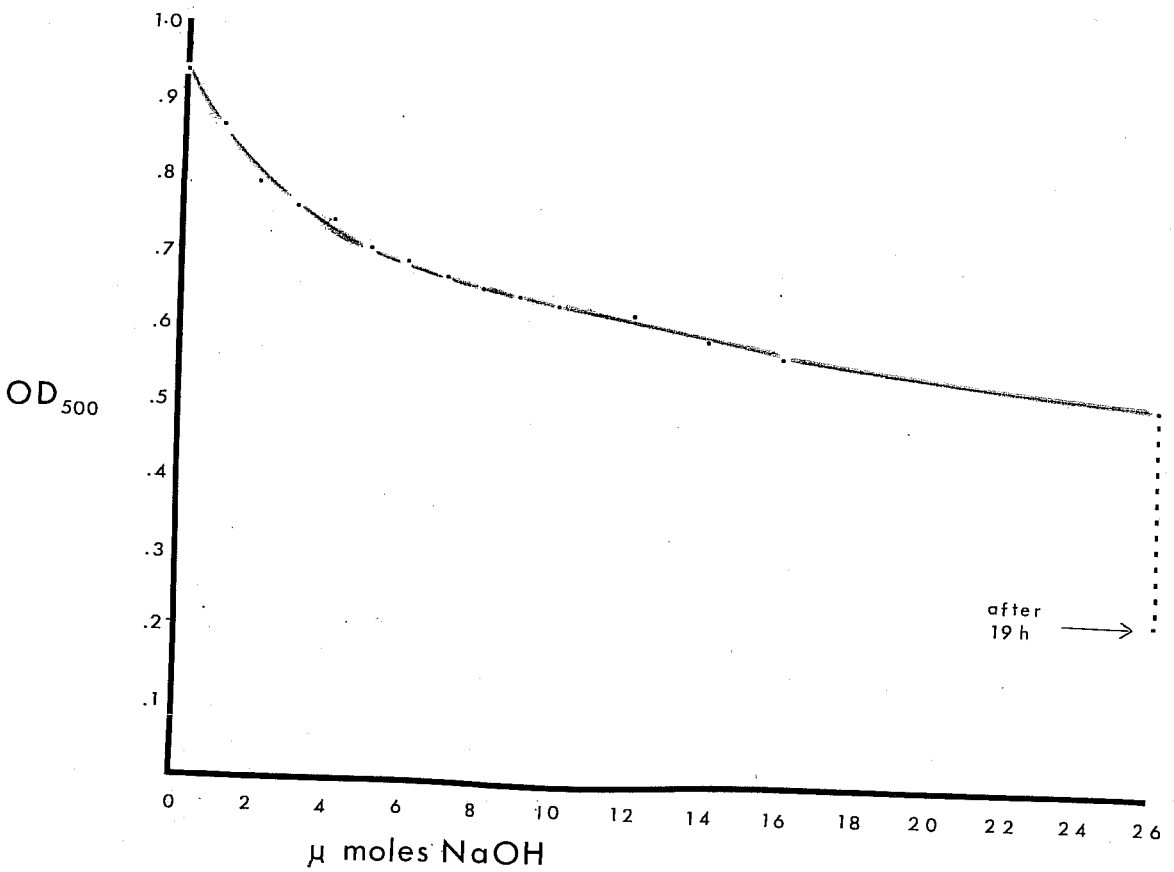


Figure 10

Solubilization of Fraction G in NaOH at 23°

After each addition of NaOH the OD_{500} was read against a water blank.

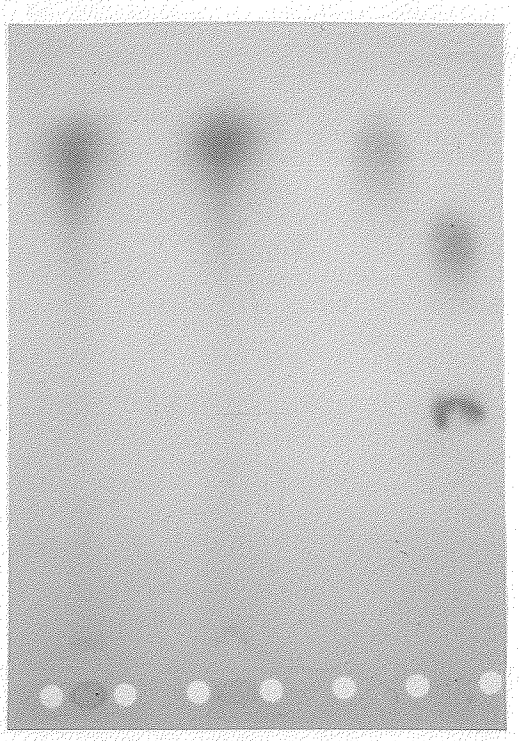


Figure 11

Cellulose Polyacetate Electrophoresis in 0.3M Cupric Acetate of 0.02N NaOH Soluble Fractions A and G

1 Fraction G

2 Fraction A

3 Standard chondroitin-4-sulfate

4 Standard hyaluronic acid, heparan sulfate

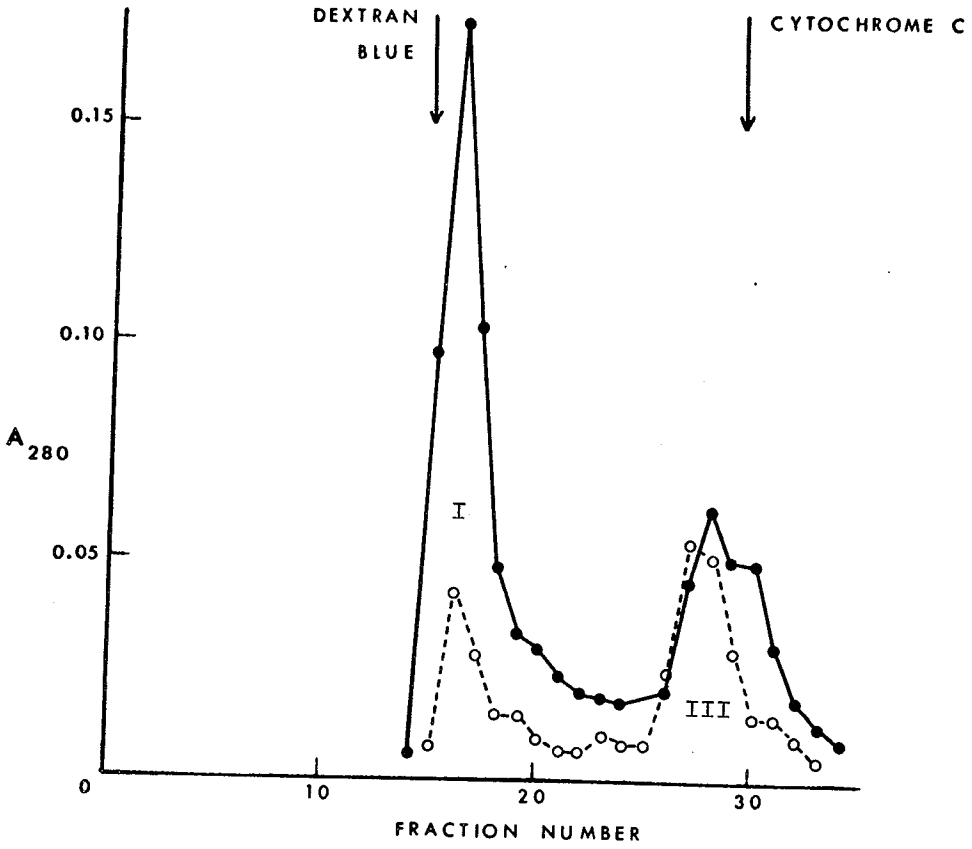


Figure 12

Gel chromatography on Sephadex G-200 of reduced and alkylated A or G in 0.1M phosphate , pH 7.0 containing 0.02% NaN_3 , 1.0% SDS, 3 mM β-ME. 4.3 ml fractions were collected.

O- - - - -O A (0.9 mg protein)

●———● G (1.6 mg protein)

A_{280} has been corrected for the absorbance of the eluant.

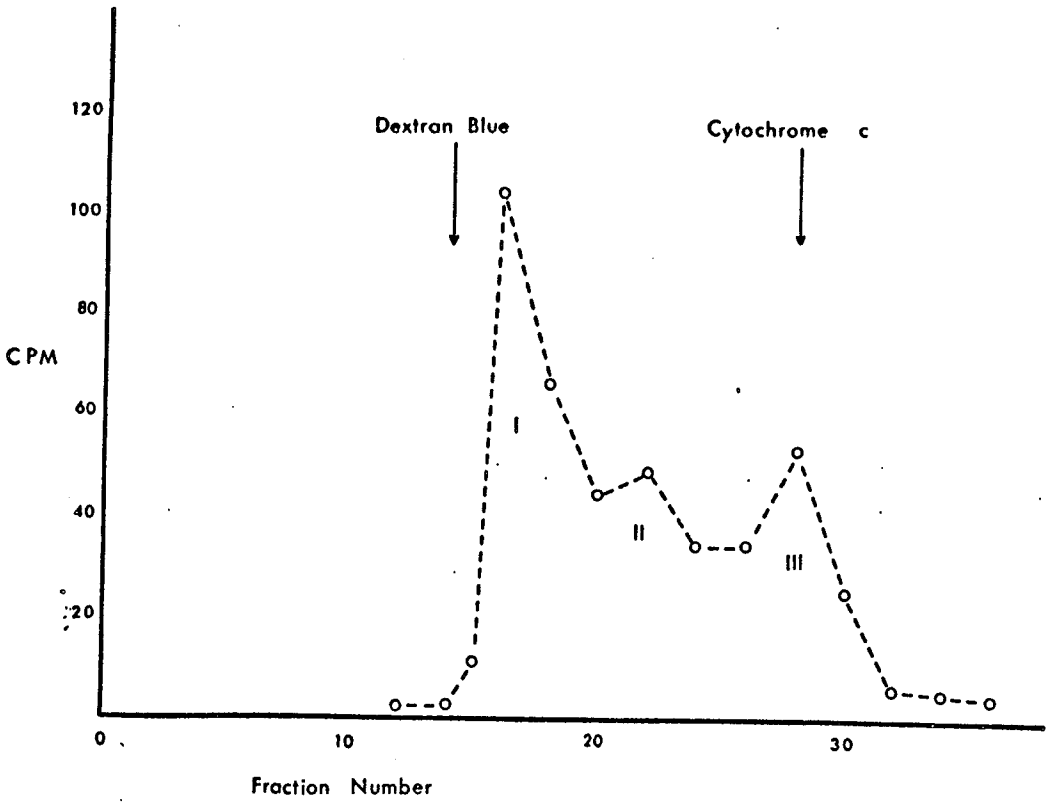


Figure 13

Gel chromatography on Sephadex G-200 of reduced and alkylated A (0.92 mg protein) in 0.1M phosphate, pH 7.0 containing 0.02% NaN_3, 1.0% SDS, 3 mM β-ME. 4.3 ml fractions were collected. CPM have been corrected for the background.

Peak I = Fractions 14 - 19 inclusive

Peak II = Fractions 20 - 25 inclusive

Peak III = Fractions 26 - 32 inclusive

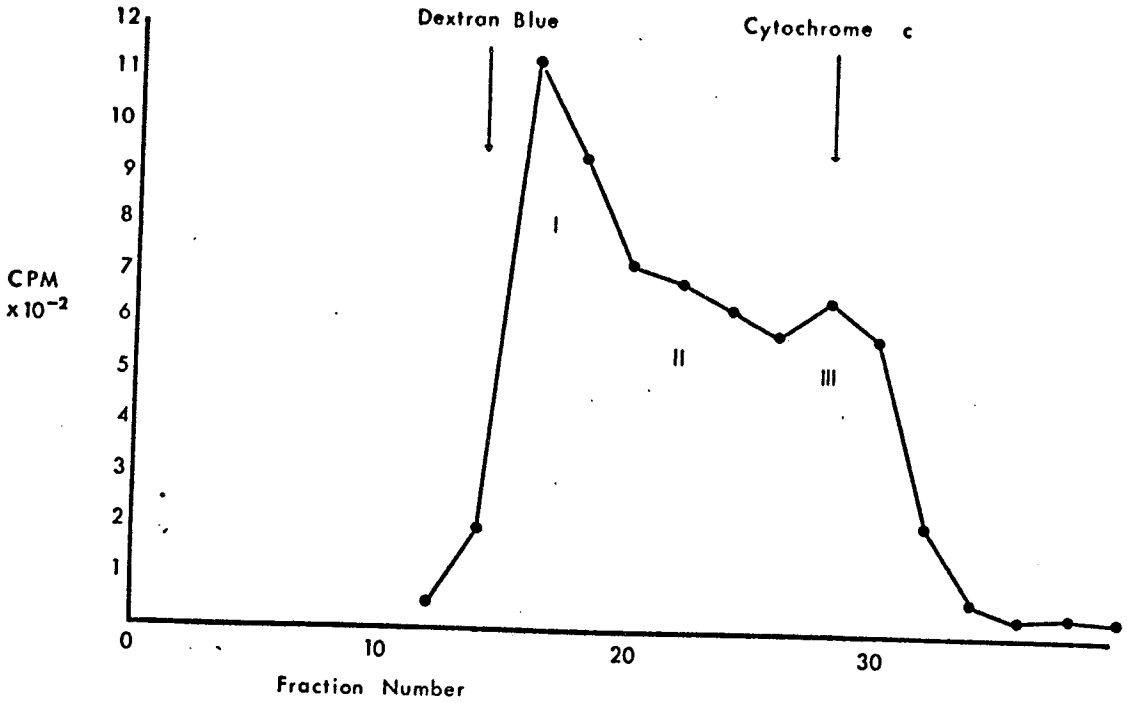


Figure 14

Gel chromatography on Sephadex G-200 of reduced and alkylated G (2.10 mg protein) in 0.1M phosphate , pH 7.0 containing 0.02% NaN_3 , 1.0% SDS , 3 mM β-ME. 4.3 ml fractions were collected. CPM have been corrected for the background.

Peak I = Fractions 12 - 19 inclusive

Peak II = Fractions 20 - 25 inclusive

Peak III = Fractions 26 - 33 inclusive

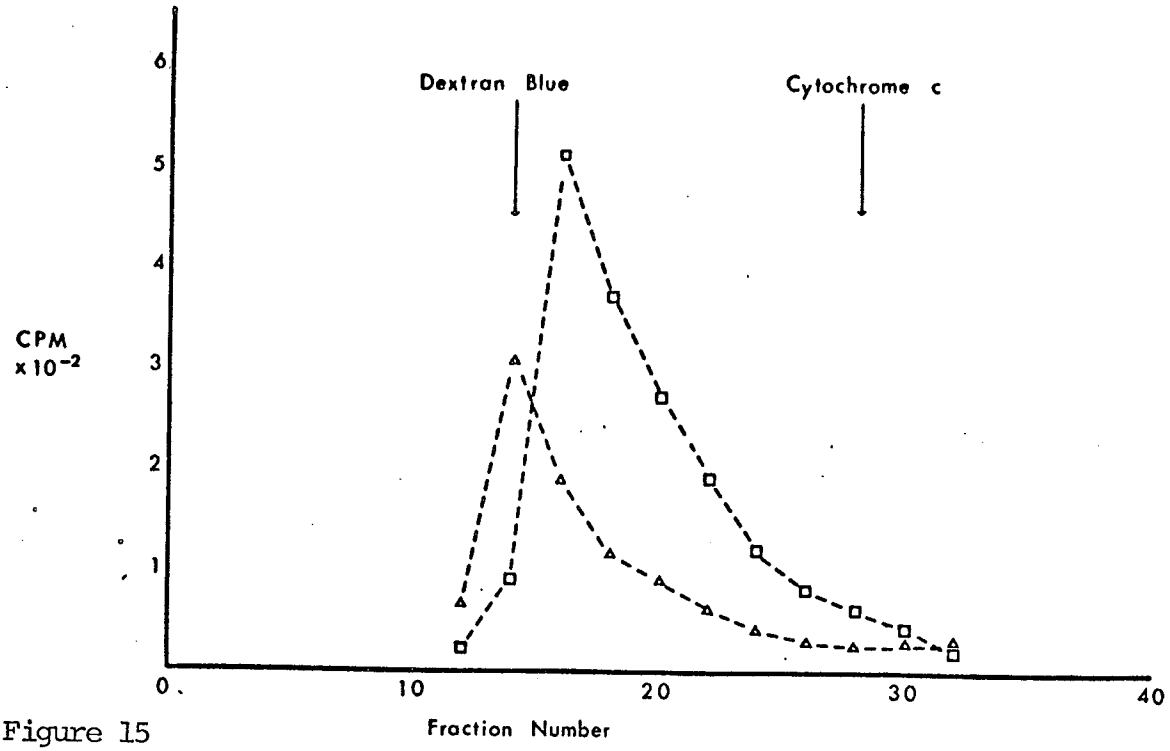


Figure 15

Gel chromatography on Sephadex G-200 of Peak I in Figure 13. 4.3 ml fractions were collected and the cpm corrected for background.

□ - - - □ Running buffer: 0.1M phosphate, pH 7.0 containing 0.02% NaN_3, 1.0% SDS, 3mM β-ME.

▽- - - -▽ Running buffer: 0.005M Tris, 0.039 glycine, pH 8.3 containing 0.02% NaN_3, 0.1% SDS, 3 mM β-ME.

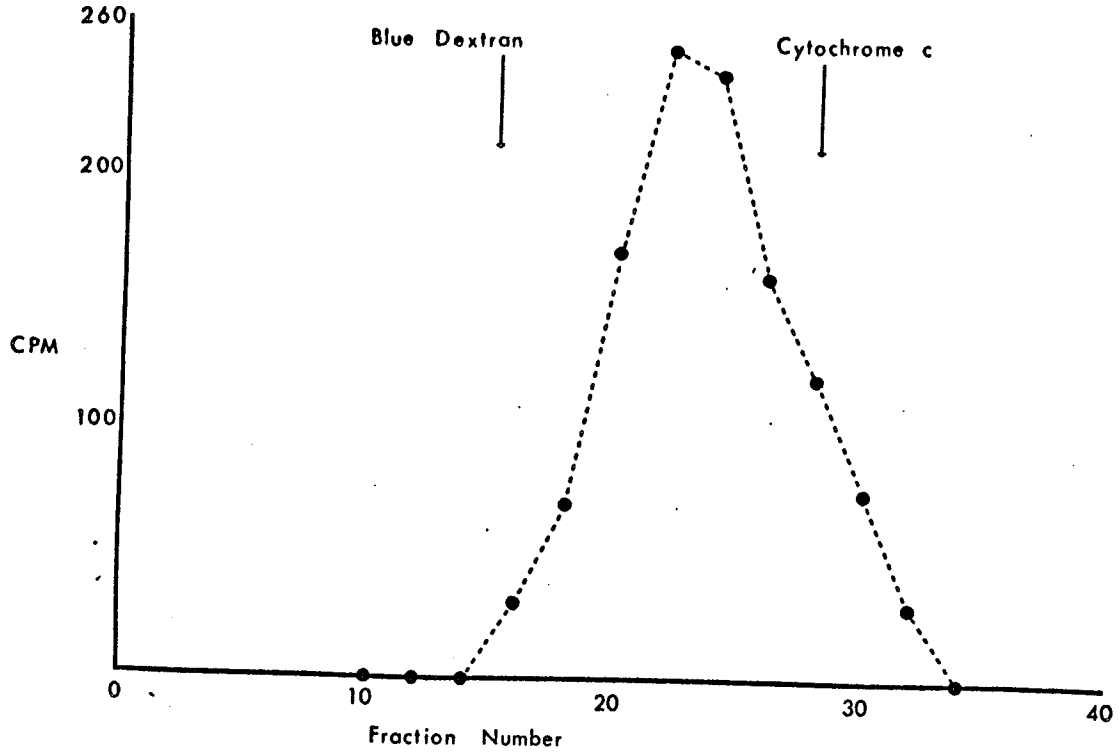


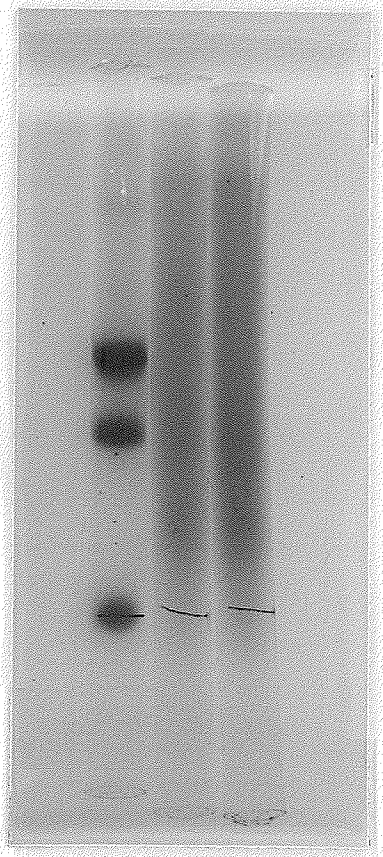
Figure 16

Gel chromatography on Sephadex G-200 of Peak II in Figure 13. 4.3 ml fractions were collected and the cpm corrected for background.

●-----● Running buffer: 0.1M phosphate, pH 7.0 containing 0.02% NaN_3, 1.0% SDS, 3mM β-ME.

Figure 17

SDS GEL ELECTROPHORESIS ACCORDING TO PROCEDURE A OF REDUCED AND ALKYLATED A OR G AND OF BOVINE SERUM ALBUMIN, OVALBUMIN AND WHALE MYOGLOBIN STANDARDS



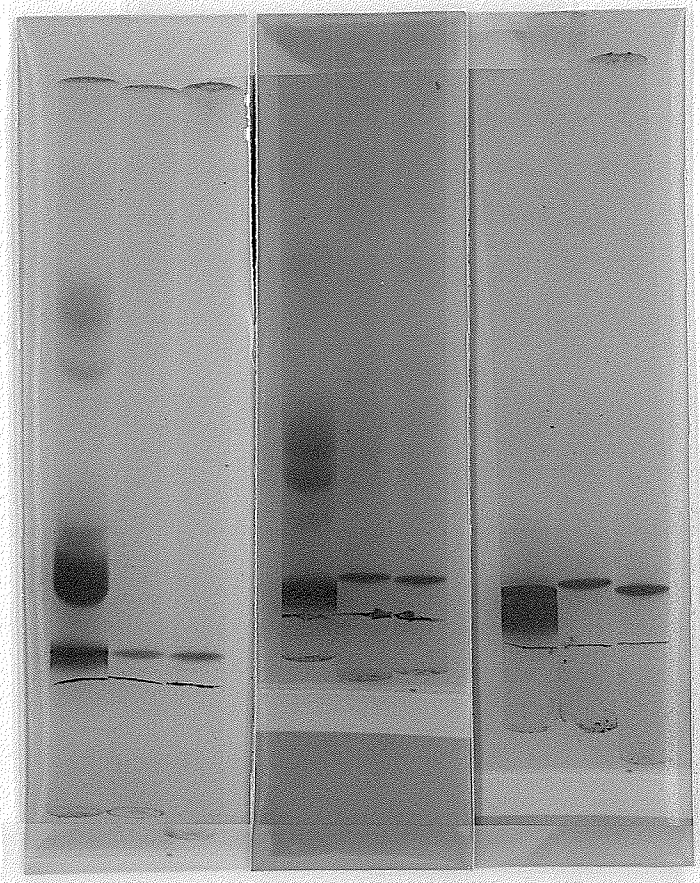
1 Standards
2 Fraction A
3 Fraction G

1, 2, & 3 were on 5% polyacrylamide in 0.1% SDS

Figure 18

SDS DISC GEL ELECTROPHORESIS ACCORDING TO PROCEDURE B

OF REDUCED AND ALKYLATED A OR G AND STANDARDS



1, 4, 7 Standards: apoferritin, bovine serum albumin, ovalbumin, whale myoglobin

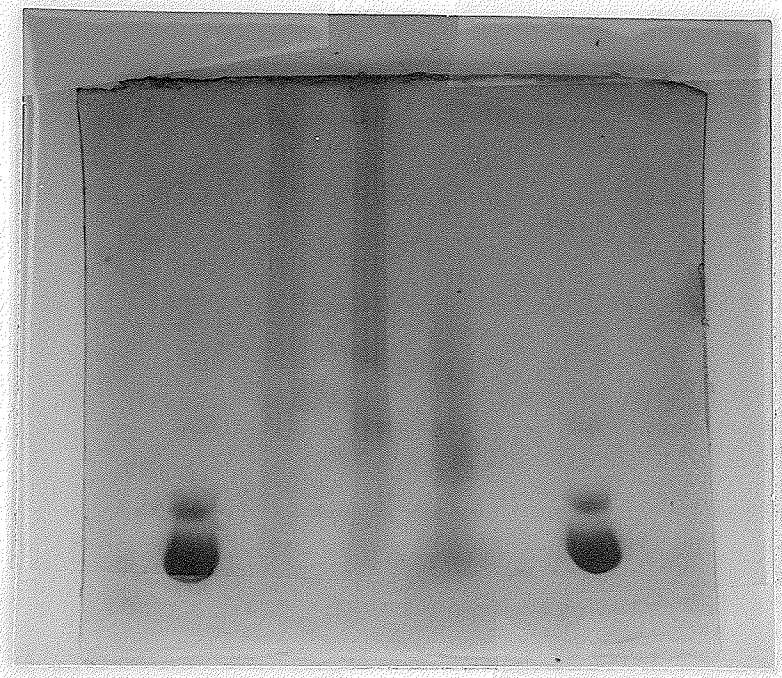
2, 5, 8 Fraction A

3, 6, 9 Fraction G

1, 2, 3 were on 5.0% polyacrylamide in 0.1% SDS
4, 5, 6 were on 3.75% polyacrylamide in 0.1% SDS
7, 8, 9 were on 2.5% polyacrylamide in 0.1% SDS

Figure 19

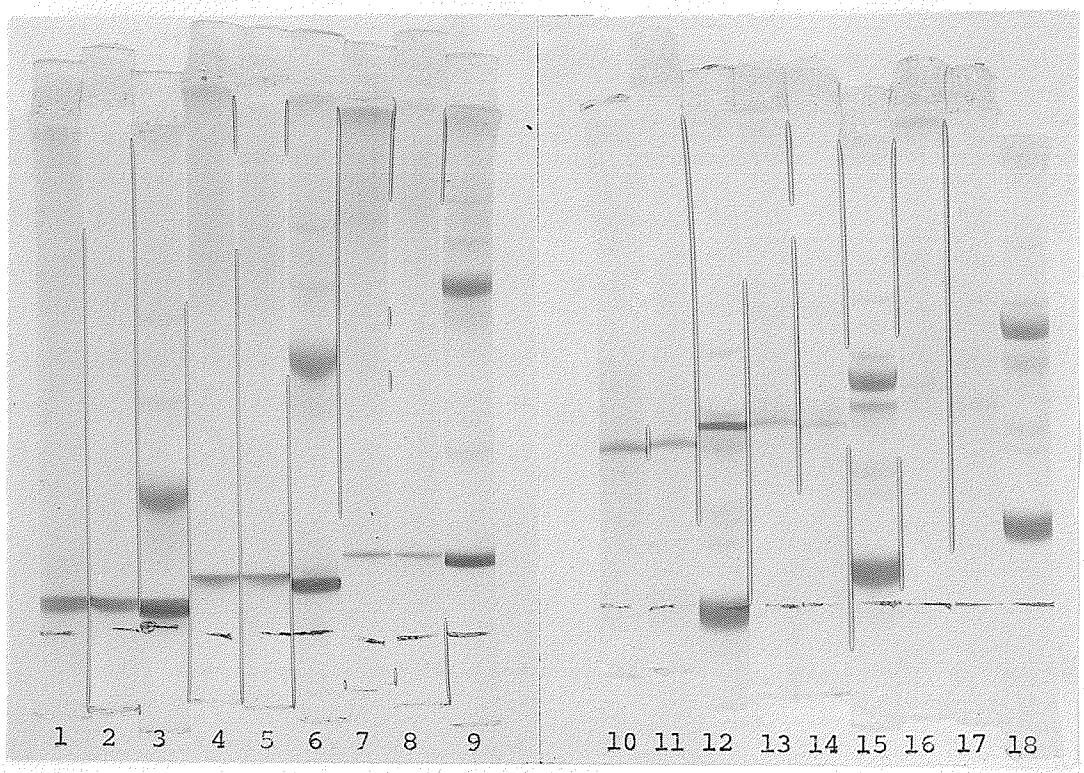
POLYACRYLAMIDE GRADIENT (2.5-27%) GEL ELECTROPHORESIS OF REDUCED AND ALKYLATED FRACTIONS ACCORDING TO PROCEDURE C



1 Cytochrome c 5µg
2 Fraction A
3 Fraction G
4 Peak III from Fraction G in Figure 12
5 Cytochrome c 5µg

Figure 20

SDS DISC GEL ELECTROPHORESIS OF REDUCED AND ALKYLATED
A OR G AND OF OVALBUMIN PLUS CYTOCHROME c STANDARDS



in 0.1% SDS in 1.0% SDS

1, 10	Fraction G	)	
2, 11	Fraction A	)	on 8% polyacrylamide
3, 12	Standards	)	
4, 13	Fraction G	)	
5, 14	Fraction A	)	on 10% polyacrylamide
6, 15	Standards	)	
7, 16	Fraction G	)	
8, 17	Fraction A	)	on 12% polyacrylamide
9, 18	Standards	)	

The applied samples were dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 1.0% SDS

In the Standard gels the upper dark band is ovalbumin and the lower cytochrome c

Figure 21

^{14}C PROFILE OF REDUCED AND ALKYLATED FRACTION A UPON ELECTROPHORESIS ON 8% POLYACRYLAMIDE IN 0.1% SDS ACCORDING TO PROCEDURE D

(Recovery = 79%)

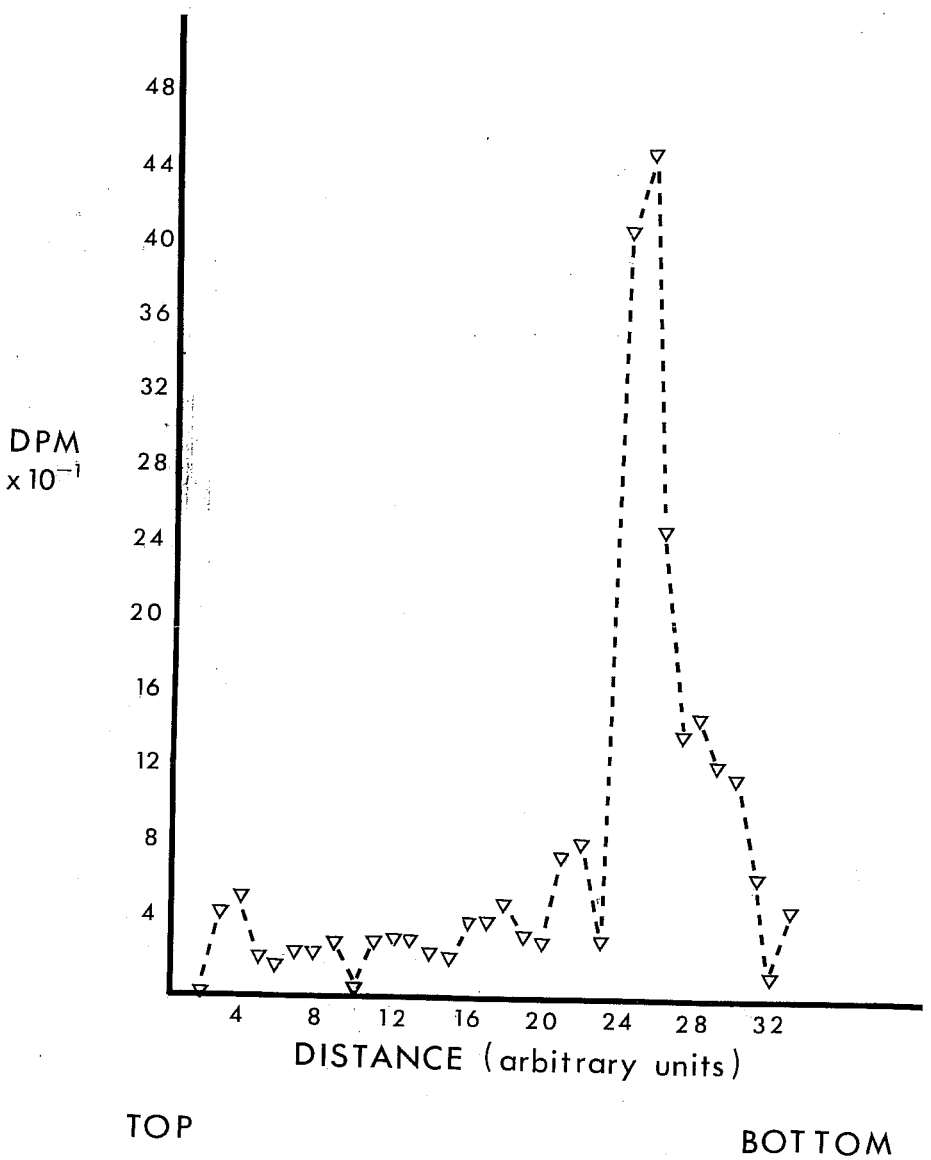


Figure 22

^{14}C PROFILE OF REDUCED AND ALKYLATED FRACTION A UPON ELECTROPHORESIS ON 12% POLYACRYLAMIDE IN 1.0% SDS ACCORDING TO PROCEDURE D

(Recovery = 76%)

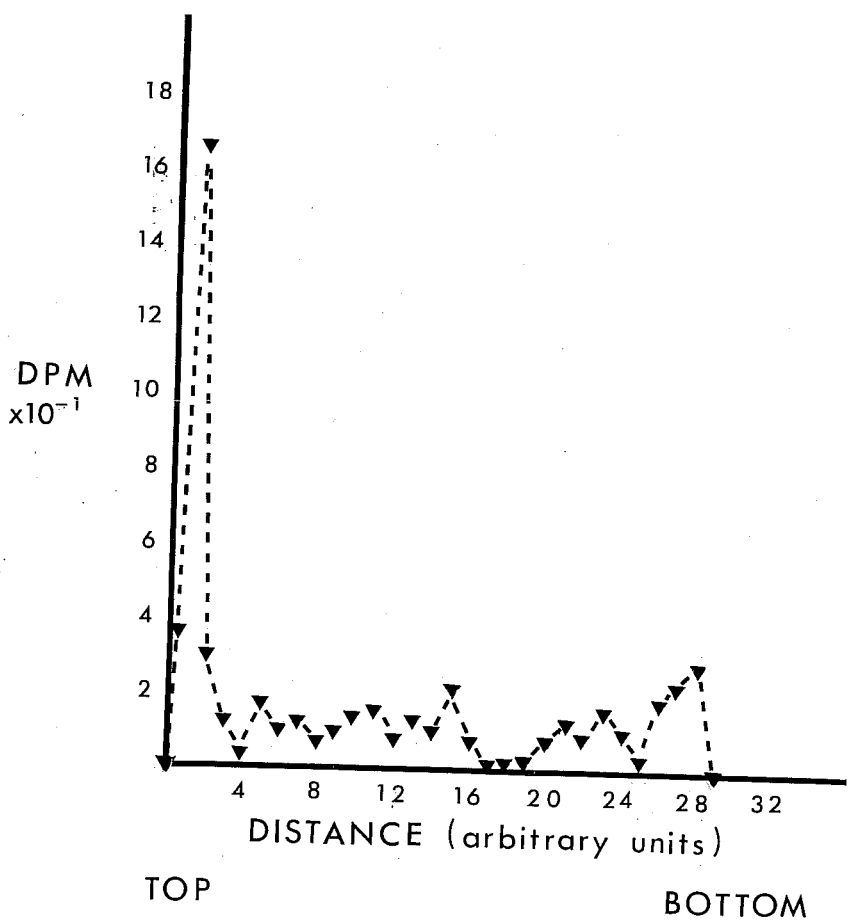
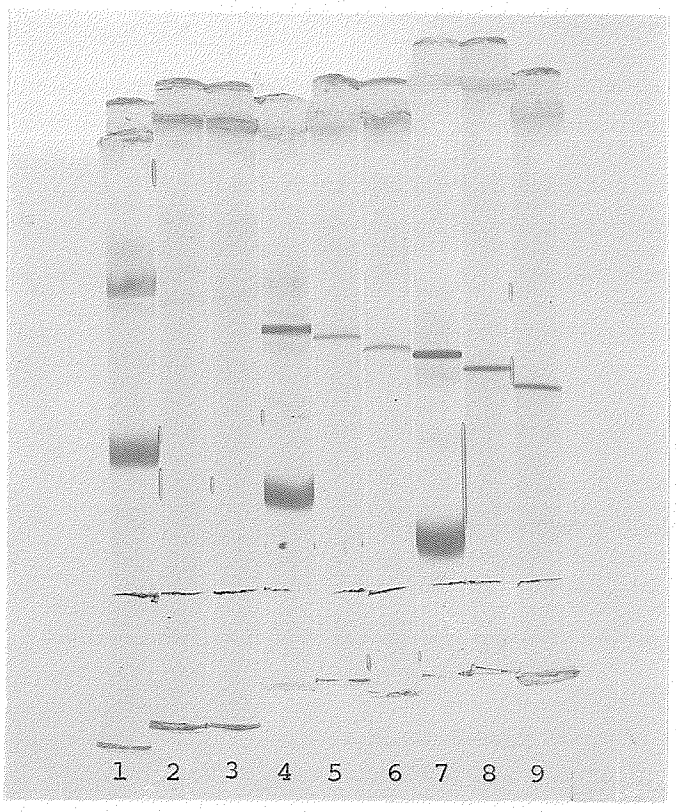


Figure 23

SDS (1.0%) 5M UREA DISC GEL ELECTROPHORESIS OF REDUCED AND ALKYLATED A OR G AND OF OVALBUMIN PLUS CYTOCHROME c STANDARDS



1	Standard	)
2	Fraction A	) on 12% polyacrylamide
3	Fraction G	)
4	Standard	)
5	Fraction A	) on 10% polyacrylamide
6	Fraction G	)
7	Standard	)
8	Fraction A	) on 8% polyacrylamide
9	Fraction G	)

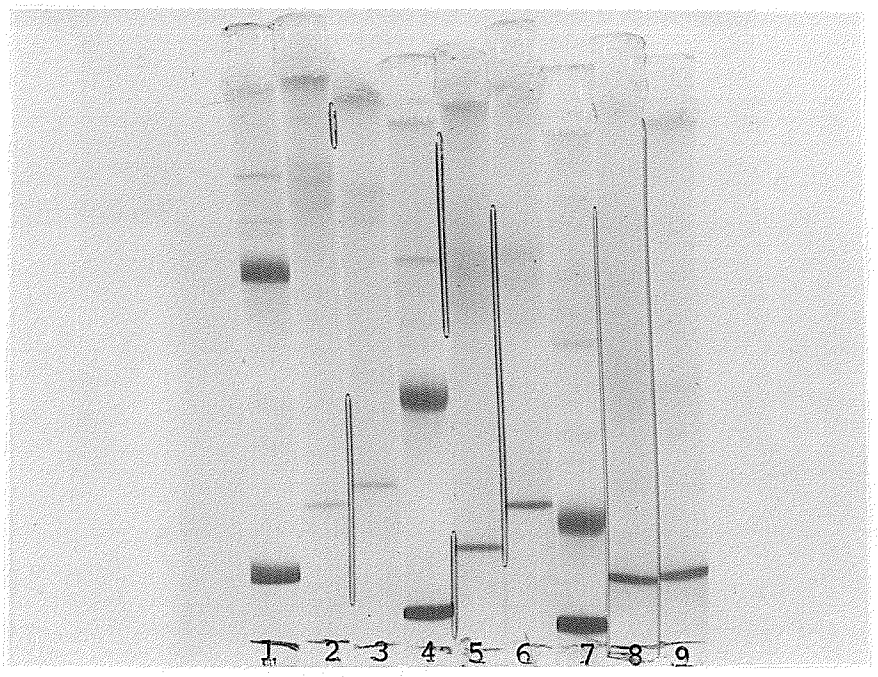
The applied samples were dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 1.0% SDS

The running gels contained 5M urea

In the standard gels the upper dark band is ovalbumin and the lower cytochrome c

Figure 24

SDS (0.1%) DISC GEL ELECTROPHORESIS OF PEAKS I & III FROM REDUCED AND ALKYLATED A AND OF OVALBUMIN PLUS CYTOCHROME c STANDARDS



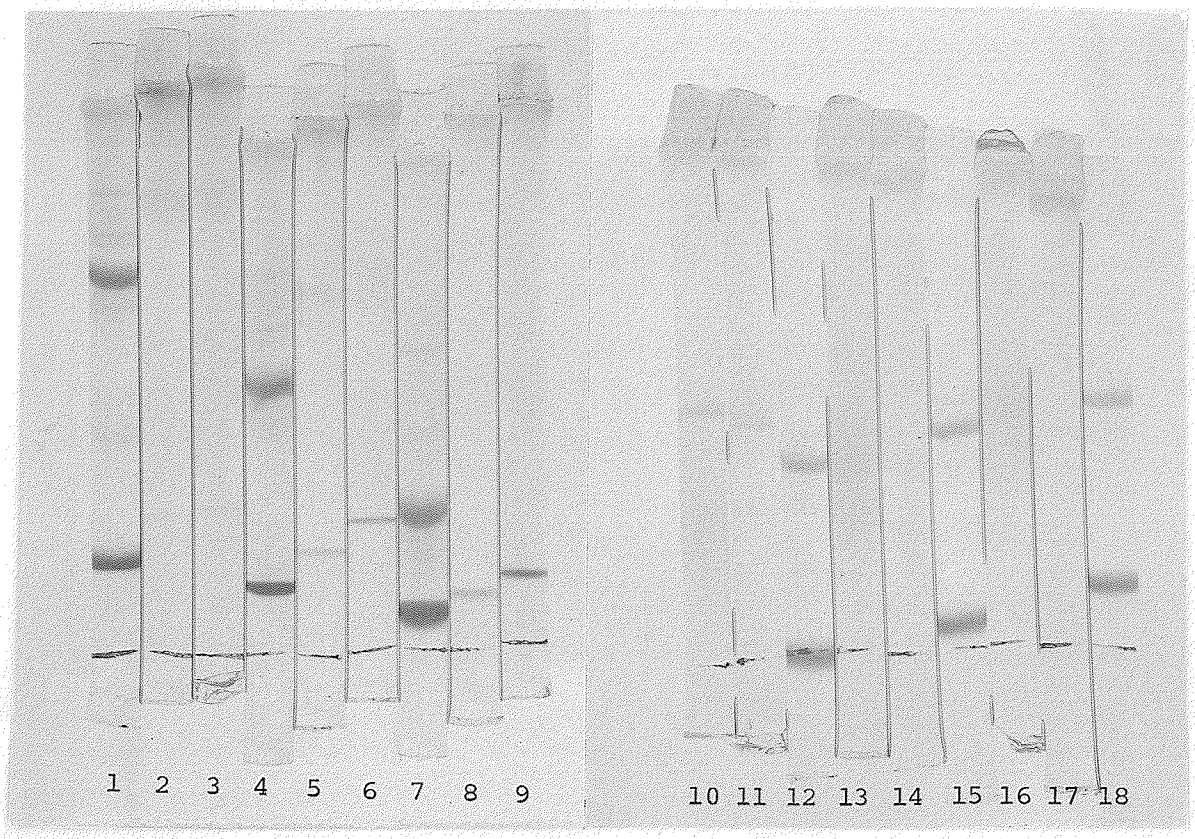
1	Standard	)	
2	Peak I	)	on 12% polyacrylamide
3	Peak III	)	
4	Standard	)	
5	Peak I	)	on 10% polyacrylamide
6	Peak III	)	
7	Standard	)	
8	Peak I	)	on 8% polyacrylamide
9	Peak III	)	

Peaks I, III were dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 5% SDS; Standards were dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 1.0% SDS

In the standard gels the upper dark band is ovalbumin and the lower cytochrome c

Figure 25

SDS DISC GEL ELECTROPHORESIS OF PEAK II FROM REDUCED AND ALKYLATED A AND G AND OVALBUMIN PLUS CYTOCHROME c STANDARDS



in 0.1% SDS in 1.0% SDS

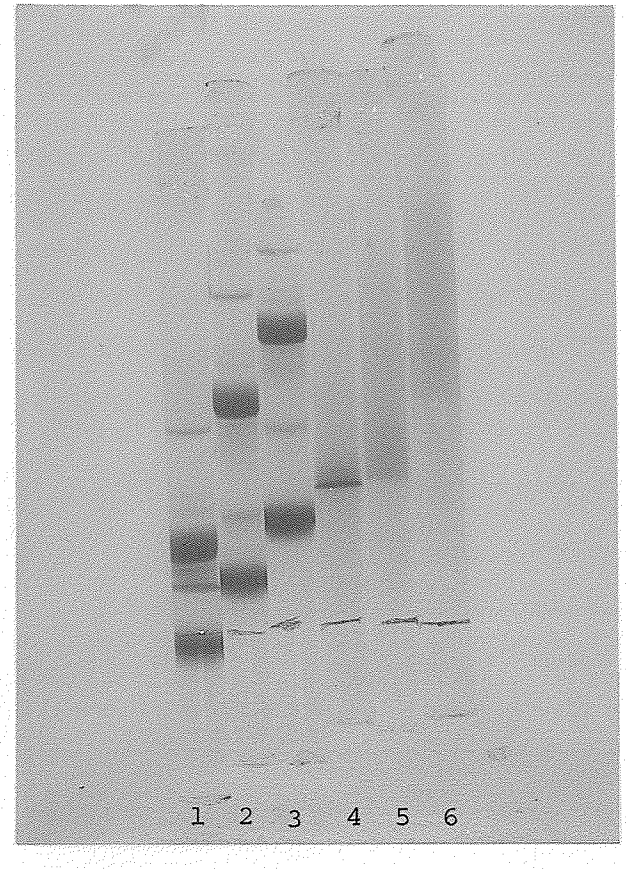
7, 12	Standard	)
8, 11	Peak II Fraction A	) on 8% polyacrylamide
9, 10	Peak II Fraction G	)
4, 15	Standard	)
5, 14	Peak II Fraction A	) on 10% polyacrylamide
6, 13	Peak II Fraction G	)
1, 18	Standard	)
2, 17	Peak II Fraction A	) on 12% polyacrylamide
3, 16	Peak II Fraction G	)

Peaks II were dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 5% SDS; Standards were dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 1.0% SDS

In the standard gels the upper dark band is ovalbumin and the lower cytochrome c

Figure 26

SDS (0.1%) DISC GEL ELECTROPHORESIS OF PEAK I FROM REDUCED AND ALKYLATED G AND OVALBUMIN PLUS CYTOCHROME c STANDARDS



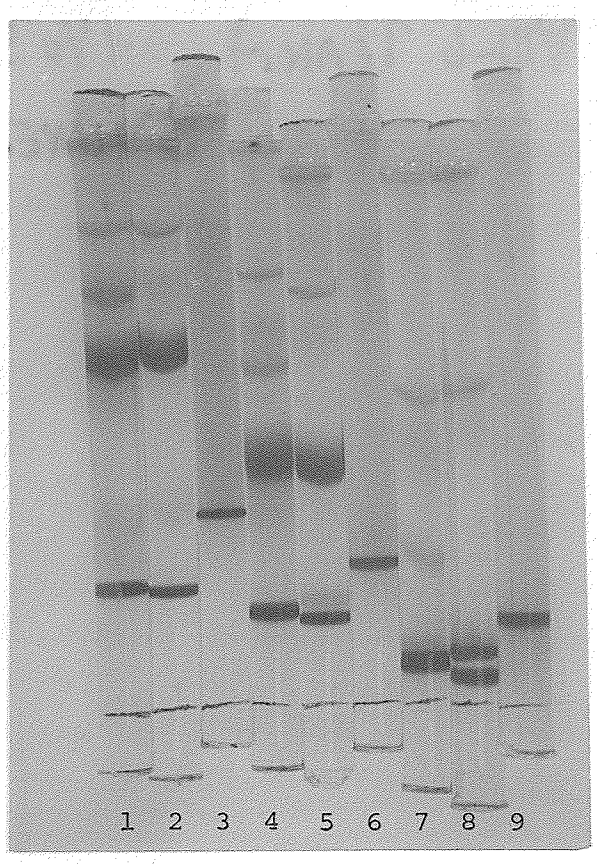
1 Standard on 8% polycarylamide
2 Standard on 10% polyacrylamide
3 Standard on 12% polyacrylamide
4 Peak I Fraction G on 8% polyacrylamide
5 Peak I Fraction G on 10% polyacrylamide
6 Peak I Fraction G on 12% polyacrylamide

Peak I was dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 5% SDS; Standards were dissolved in 0.062M Tris-HCl (pH 6.7) containing 1.0% SDS

In the standard gels the upper dark band is ovalbumin and the lower cytochrome c

Figure 27

SDS (0.1%) 5M UREA DISC GEL ELECTROPHORESIS OF SEPHADEX G-200 PEAK I FROM REDUCED AND ALKYLATED G AND OVALBUMIN PLUS CYTOCHROME c STANDARDS



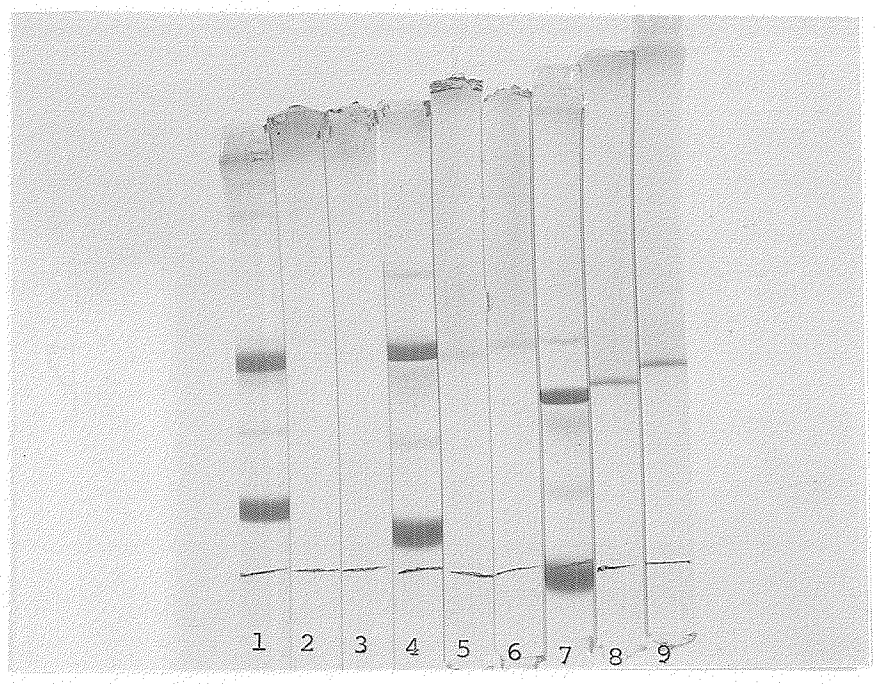
2	Standard	) on 12% polyacrylamide
3	Peak I Fraction G	)
5	Standard	) on 10% polyacrylamide
6	Peak I Fraction G	)
8	Standard	) on 8% polycarylamide
9	Peak I Fraction G	)

Peak I was dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 5% SDS; Standards were dissolved in 0.062M Tris-HCl (pH 6.7) containing 1% SDS

In the standard gels (2, 5, 8) upper dark band is ovalbumin and the lower cytochrome c

Figure 28

SDS (1.0%) DISC GEL ELECTROPHORESIS OF PEAK I FROM REDUCED AND ALKYLATED G AND OVALBUMIN PLUS CYTOCHROME c STANDARDS



1	Standard	)
2	Peak I Fraction G	) on 12% polycarylamide
3	Peak I Fraction G	)
4	Standards	)
5	Peak I Fraction G	) on 10% polyacrylamide
6	Peak I Fraction G	)
7	Standard	)
8	Peak I Fraction G	) on 8% polyacrylamide
9	Peak I Fraction G	)

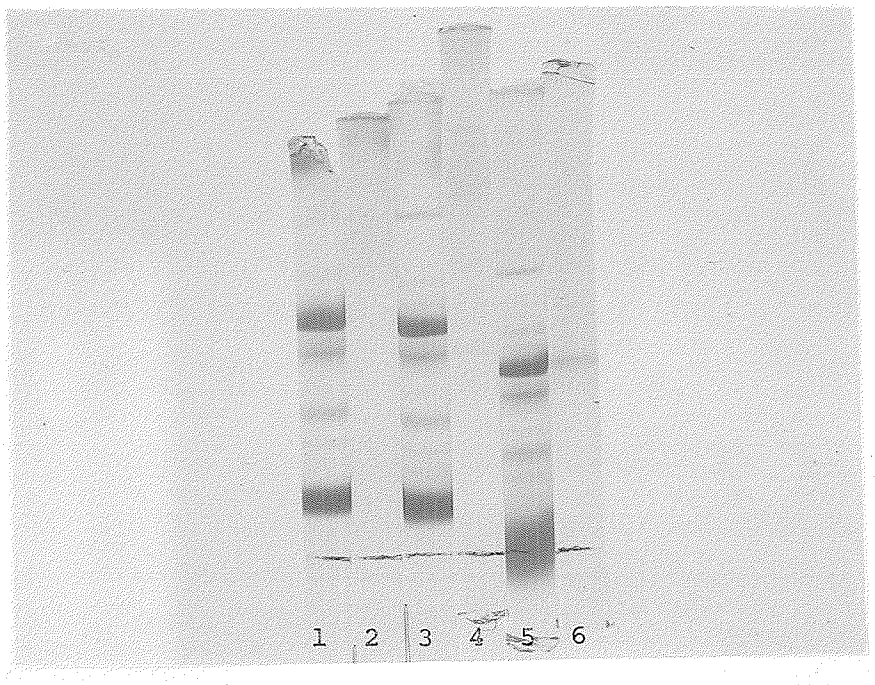
In the standard gels the upper dark band is ovalbumin and the lower cytochrome c

In gels 2, 5, 8 Peak I was dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 5% SDS

In gels 3, 6, 9 Peak I was dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 5% SDS and heated to 100° for 3 min

Figure 29

SDS (5.0%) DISC GEL ELECTROPHORESIS OF PEAK I FROM
REDUCED AND ALKYLATED G AND OVALBUMIN
PLUS CYTOCHROME c STANDARDS



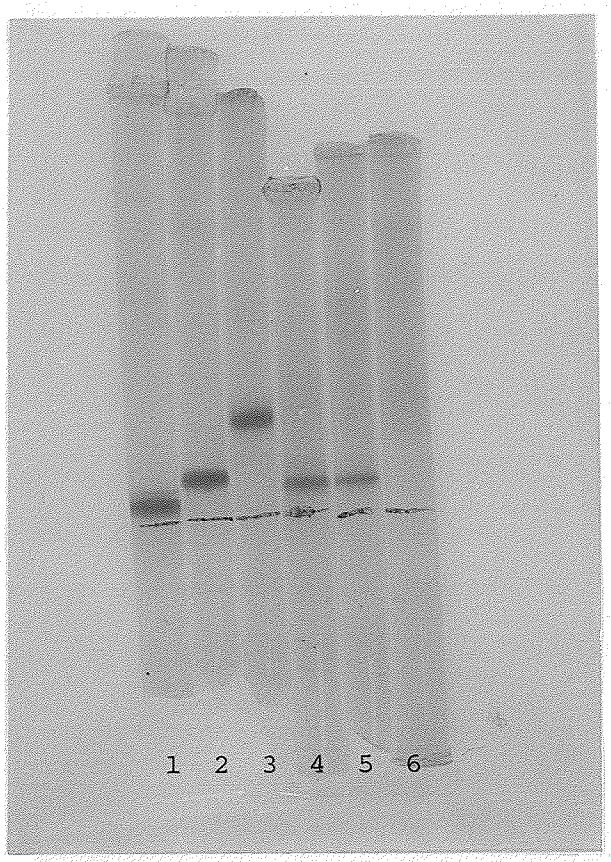
1	Standard	on 12% polyacrylamide
2	Peak I Fraction G	
3	Standard	on 10% polyacrylamide
4	Peak I Fraction G	
5	Standard	on 8% polyacrylamide
6	Peak I Fraction G	

Peak I was dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 5% SDS; Standards were dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 1% SDS

In the standard gels the upper dark band is ovalbumin and the lower cytochrome c

Figure 30

SDS (0.1%) DISC GEL ELECTROPHORESIS OF PEAK III FROM REDUCED AND ALKYLATED G AND CYTOCHROME c STANDARD



1	Standard	on 8% polyacrylamide
2	Standard	on 10% polyacrylamide
3	Standard	on 12% polyacrylamide
4	Peak III Fraction G	on 8% polyacrylamide
5	Peak III Fraction G	on 10% polyacrylamide
6	Peak III Fraction G	on 12% polycarylamide

Peak III was dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 5% SDS; Standard was dissolved in 0.062M Tris-HCl buffer (pH 6.7) containing 1.0% SDS

Figure 31

SEDIMENTATION VELOCITY MEASUREMENTS ON FRACTION A

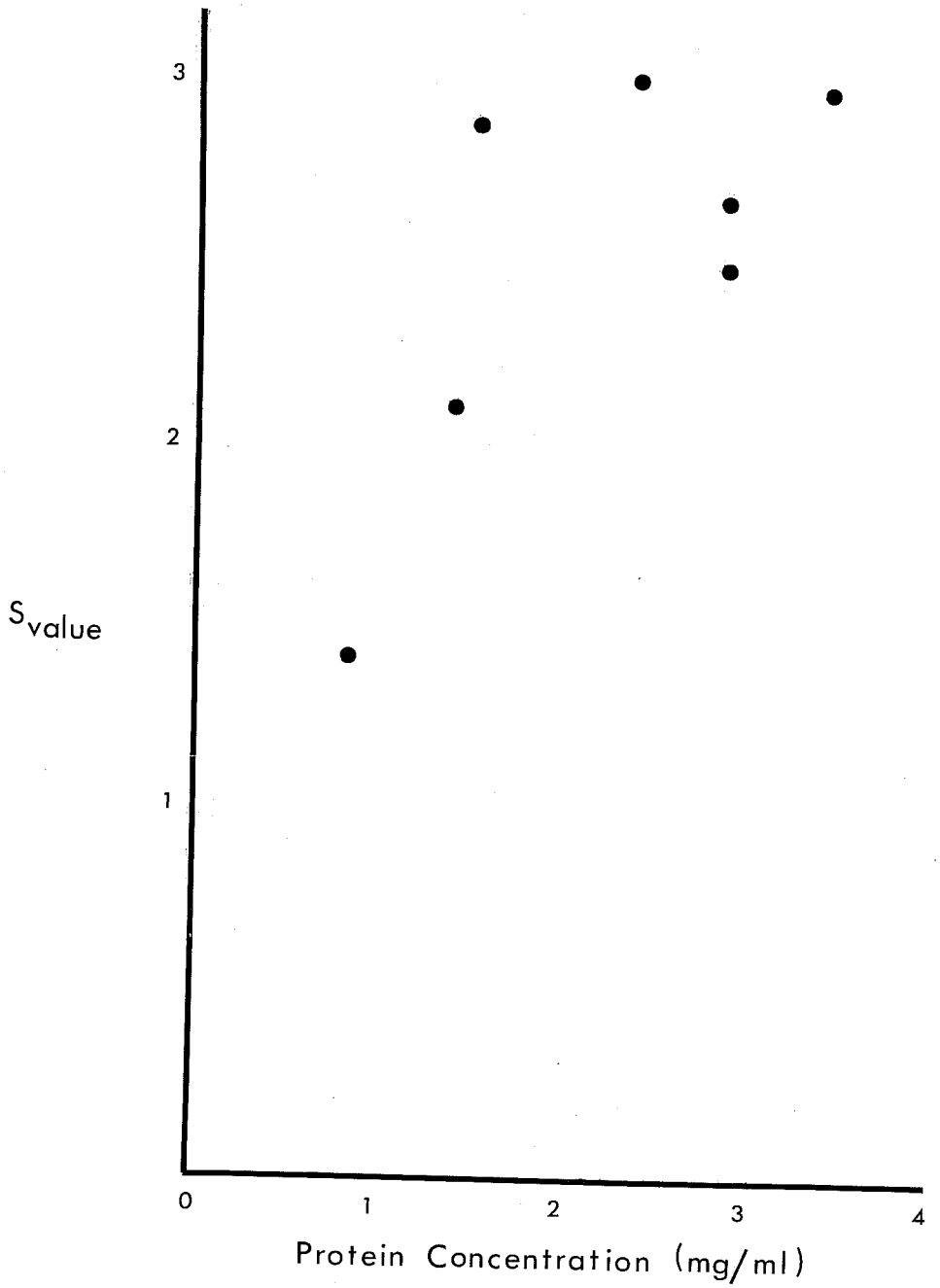


Figure 32

SEDIMENTATION VELOCITY MEASUREMENTS ON FRACTION G

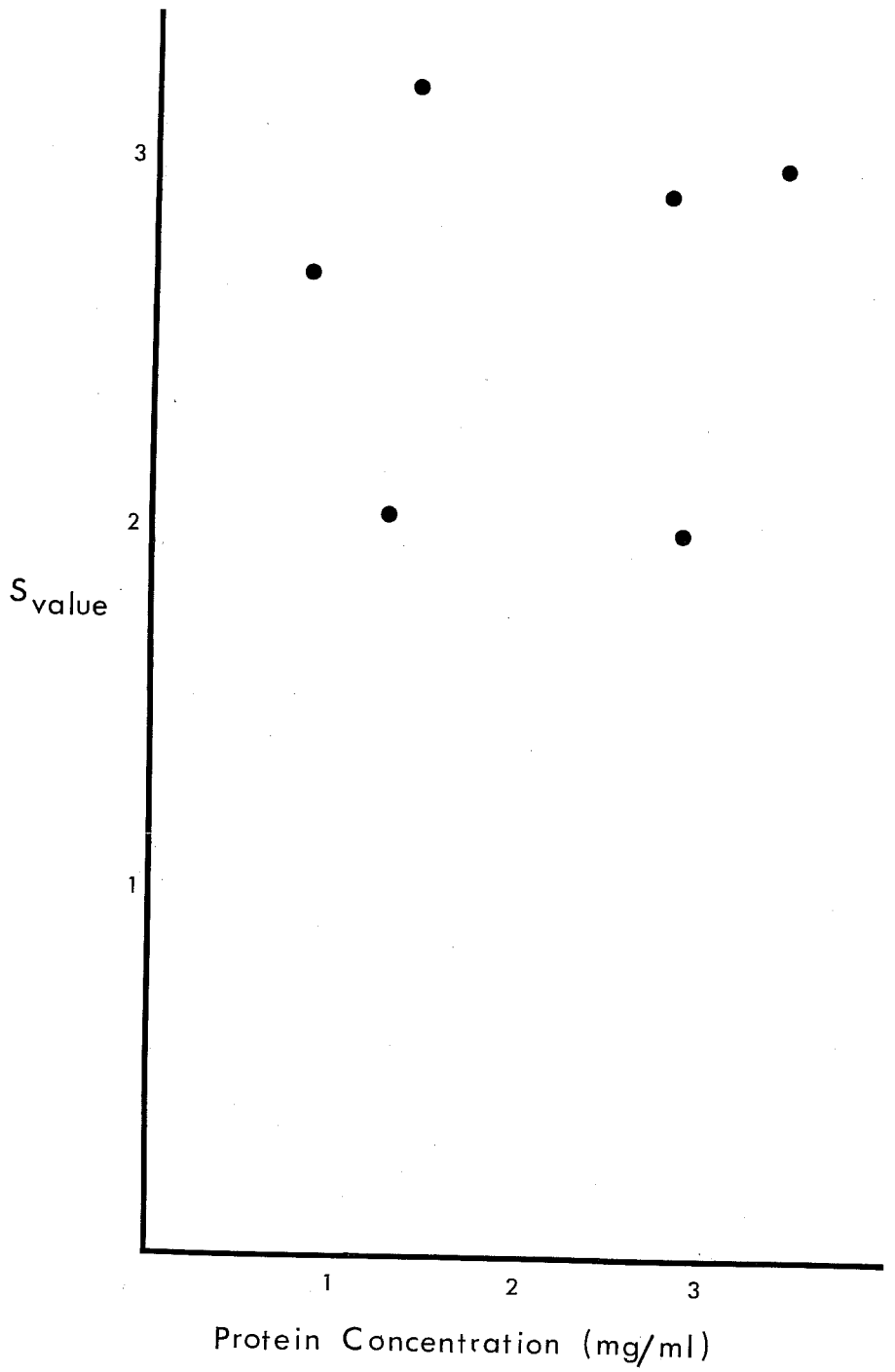
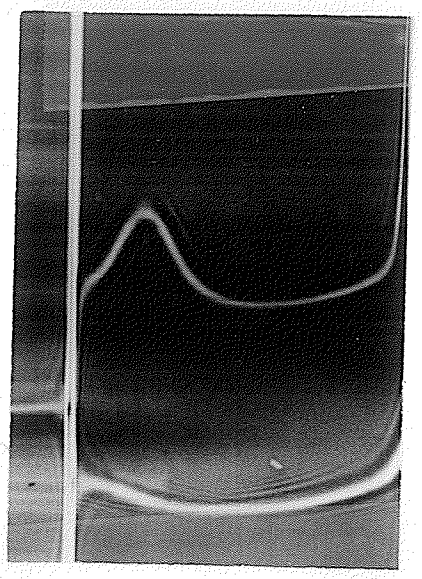
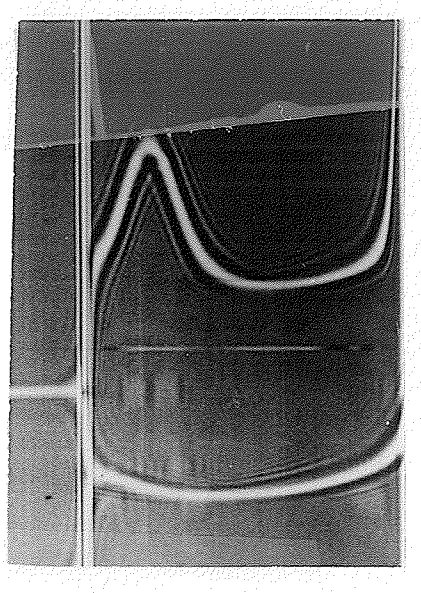


Figure 33

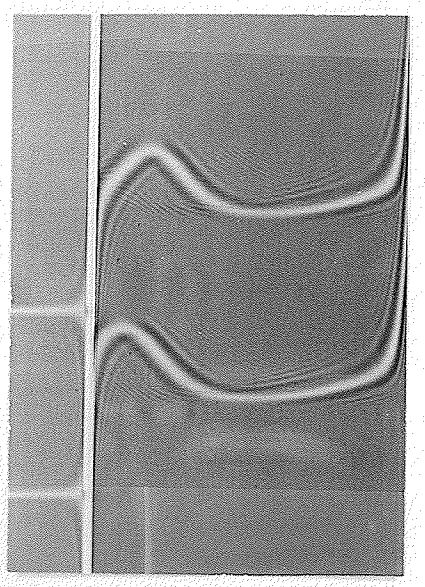
SCHLIEREN PATTERNS OBTAINED FOR FRACTIONS A AND G



(a)



(b)

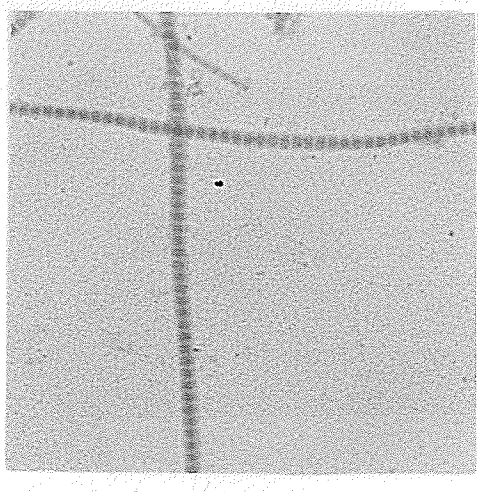


(c)

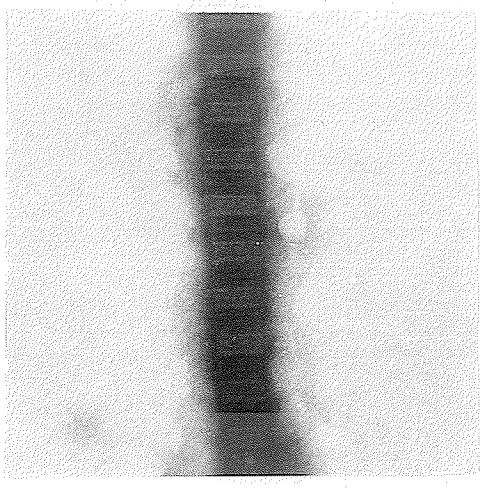
(a) Fraction A 2.36mg/ml in 0.1% SDS (S=3.03)

(b) Fraction G 2.77mg/ml in 0.1% SDS (S=2.91)

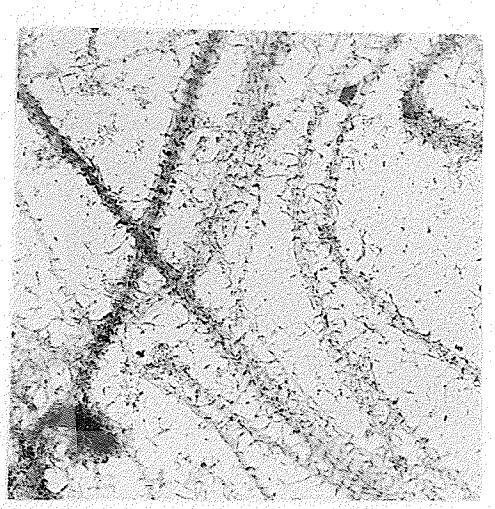
(c) Fraction G (upper) 0.83mg/ml in 0.1% SDS (S=2.69)
Fraction A (lower) 0.83mg/ml in 0.1% SDS (S=1.43)



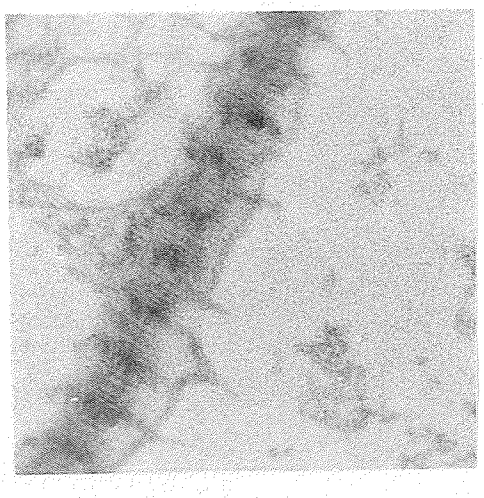
(a)



(b)



(c)

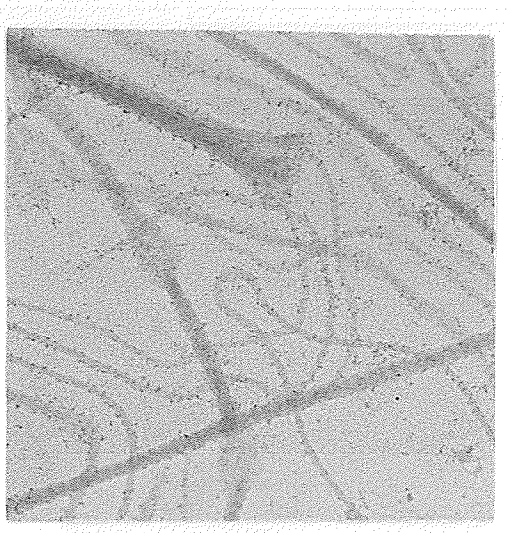


(d)

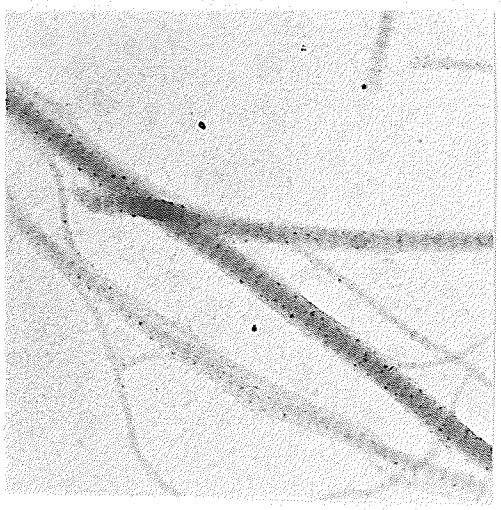
Figure 34 ELECTRON MICROGRAPHS OF INSOLUBLE CARTILAGE FRACTIONS

(a,b) Fraction 1, with PTA; (c,d) Fraction 1, with Alcian + PTA

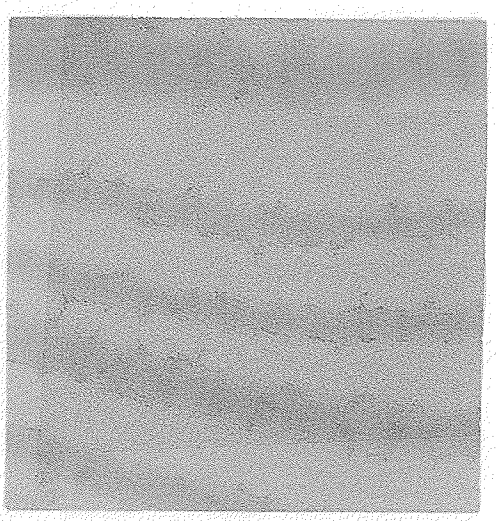
(a,c) x 26,700; (b,d) x 95,600



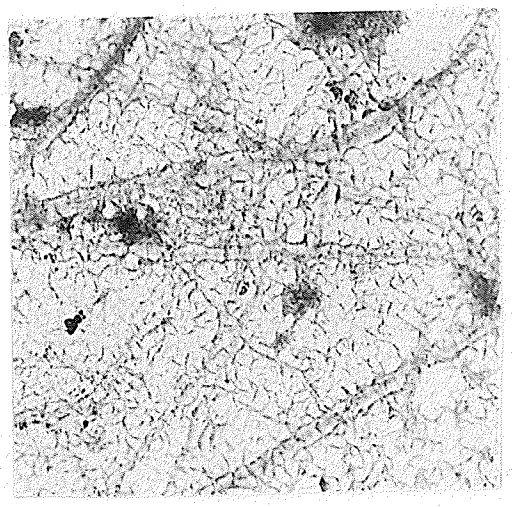
(a)



(b)



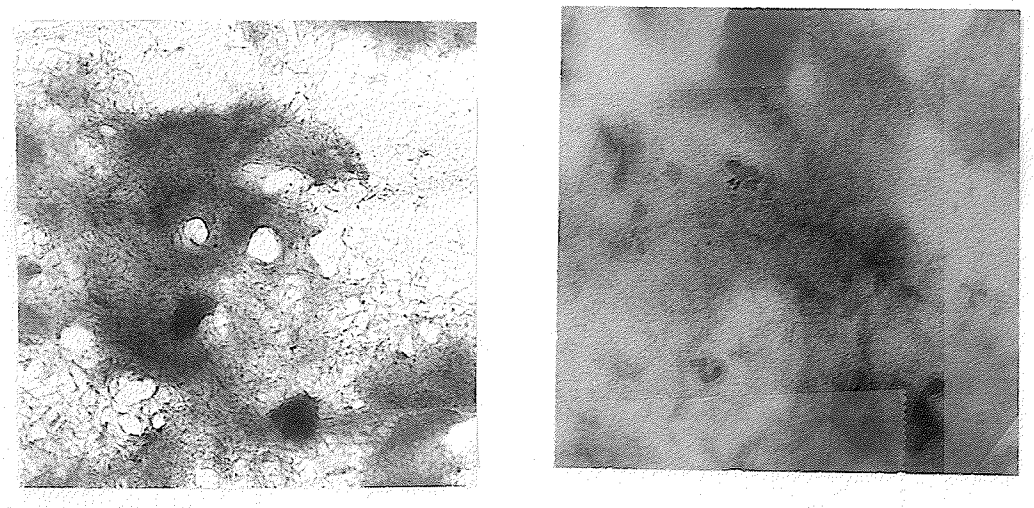
(c)



(d)

Figure 35 ELECTRON MICROGRAPHS OF INSOLUBLE CARTILAGE FRACTIONS

(a) Fraction 1, with lead acetate; (b) Fraction 1, with ruthenium red (c) Fraction 1, with lead hydroxide; (d) Fraction 1 + proteoglycan, with alcian + PTA. (a,b,d) x 26,700; (c) x 95,600



(a) (b)

Figure 36 ELECTRON MICROGRAPHS OF INSOLUBLE CARTILAGE FRACTIONS

(a) Fraction A, with alcian + PTA x 26,700

(b) Fraction A, with alcian + PTA x 95,600

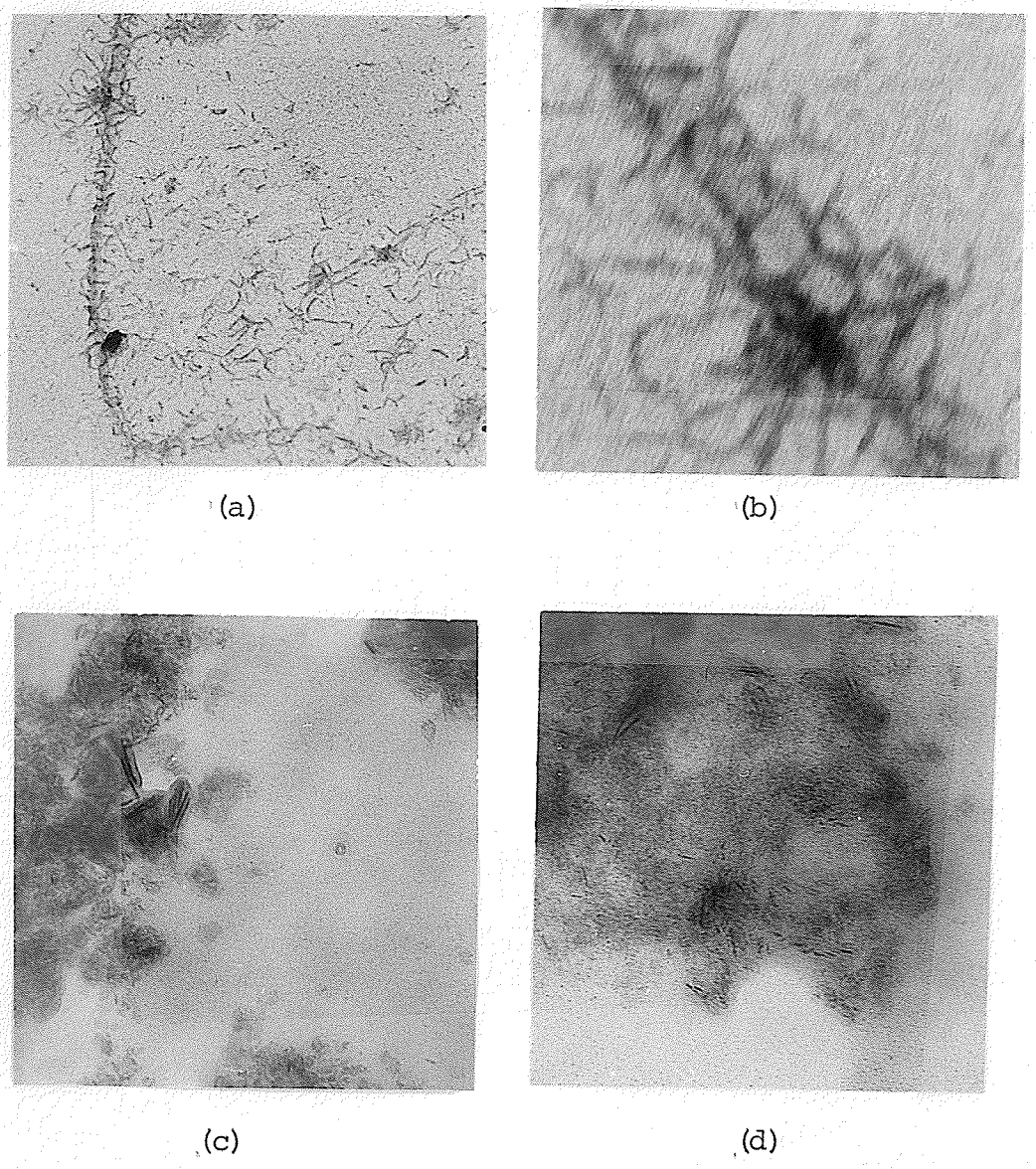


Figure 37 ELECTRON MICROGRAPHS OF INSOLUBLE CARTILAGE FRACTIONS

(a,b) Fraction G, with alcian + PTA; (c) Fraction G, with lead acetate; (d) Fraction G, with ruthenium red

(a) x 26,700; (b,c,d) x 95,600

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