SOME STUDIES ON THE PROTEOGLYCAN, PP-L, FROM BOVINE NASAL CARTILAGE

A Thesis

presented by

TIMOTHY JOHN PETERS, M.B., Ch.B.

in application for the Degree of Master of Science in

The University of St. Andrews

1967

CONTENTS

DECLARATION

I hereby declare that the following thesis is a record of the results of experiments carried out by me. It is my own composition and it has not previously been presented in application for a Higher Degree.

The experiments were carried out in the Department of Biochemistry, University of St. Andrews, under the supervision of Professor G. R. Tristram.

CERTIFICATE

I hereby certify that Timothy John Peters has spent four terms in Research Work under my supervision and that he has fulfilled the conditions of Ordinance No. (51) (St. Andrews) and that he is qualified to submit the accompanying thesis in application for the degree of Master of Science.

UNIVERSITY CAREER

I entered the University of St. Andrews as an undergraduate in the Faculty of Medicine in October 1958, and graduated, with honours, M.B., Ch.B., in June 1964.

This work was carried out during the period from October 1965 to October 1966 when enrolled as a post-graduate student in the Department of Biochemistry, St. Salvator's College, University of St. Andrews.

ACKNOWLEDGMENTS

I am grateful to Professor G. R. Tristram for his supervision, and to Dr. A. Serafini-Fracassini for much guidance and helpful discussion during this work.

I am grateful to Mrs. L. Serafini-Fracassini for the amino acid analyses, to Dr. J. W. Smith of the Department of Anatomy for help with the electron microscopy, to Dr. D. Thirkell for help with the infra-red spectroscopy and to all other members of the Department for their helpful discussions and advice.

I wish to thank Mrs. McAlister for typing this thesis and Mr. E. Carstairs for the photography.

"Science has but one language,
that of quantity, and but one
argument, that of experiment."

Ernest Henry Starling (1866-1927)

I. INTRODUCTORY DISCUSSION

Until recently, the interest shown in the nature of the ground substance of connective tissue as compared to that shown in the fibrillary elements collagen and elastin, was very scanty. Indeed, the histological description 'amorphous', which is applied to the ground substance emphasises the lack of interest of successive generations of microscopists. However, over the past thirty years much progress has been made in analytical and structural studies of the carbohydrate moieties of the ground substance. Once it had been realised that the proteins isolated with the carbohydrates were not just contaminants, the studies of the proteinpolysaccharide complexes, which have slowly been gathering momentum over the past decade, could begin in earnest.

In this review it is proposed to discuss some of the studies carried out on the inter-fibrillary ground substance, with special reference to the macromolecular protein-polysaccharide complexes. Following a historical review, some of the problems of nomenclature will be considered. The rest of the discussion will be on the proteinpoly-saccharide complexes of mammalian cartilage, predominantly

nasal cartilage: their structure, properties and metabolism.

a) Historical Review

By the beginning of the nineteenth century it was believed that all cartilaginous tissues had the same organic composition as tendon and bone; all yielding glue on boiling with water (Berzelius, 1818). However, Müller (1837) showed that the 'glue' produced from cartilage differed from that produced by tendon, skin and bone in yielding a precipitate with acetic acid. The term 'mucin' was coined by Scherer (1846) (who worked predominantly on epithelial secretions), as 'Schleimstoff', who showed it to be of a different composition from protein. Eichwald (1865) showed that mucin on hydrolysis gave a substance which reduced Fehling's solution. Even to this day pathologists believe that mucoproteins, by definition, are precipitated by dilute acetic acid, cf. the pseudo-mucin of ovarian tumours which is not precipitated by acetic acid.

Studies on cartilage led to the belief that this tissue yielded solutions of 'chondrin' on boiling with water, whereas skin and tendon yielded solutions of collagen, i.e. gelatin.

Parallel to the studies on the composition of various epithelial mucins carried out by several workers in the latter half of the nineteenth century, similar studies were carried out on 'chondrin', leading to the isolation of chondroitin sulphuric acid by Krukenberg (1884). Krukenberg believed that this substance was linked to protein in the cartilage by a salt linkage, as extraction of the cartilage with alkali broke these linkages giving appreciable yields, whereas neutral or acid extractions were relatively ineffective. Until recently all attempts at extracting mucopolysaccharides from tissues have been aimed at getting as protein-free material as possible. Thus, for many years, probably the most important component of ground substance, the non-collagenous protein, has been discarded.

The various isolation procedures advocated, such as enzymic deproteinisation (Schmiedeberg, 1891), extraction with potassium chloride and potassium carbonate (Einbinder and Schubert, 1950) or with calcium chloride (Meyer and Smyth, 1937) were all designed to remove the protein which was tacitly regarded as a contaminant. Mörner (1889) was probably the first to intentionally isolate a protein-polysaccharide complex which he called 'chondromucoid';

it was isolated from tracheal cartilage. During the next sixty years the literature contains very few references to work on chondromucoproteins until the studies of Schubert and his colleagues, in the last decade, on the isolation of proteinpolysaccharide complexes of bovine nasal cartilage.

By the end of the nineteenth century the main components of the acid mucopolysaccharides were known to be glucuronate, glycosamine, sulphate and acetate (Schmiedeberg, 1891) and, following the temporary decline of interest in the mucoproteins of blood and epithelial secretions, much work was done on the isolation and characterisation of the various acid mucopolysaccharides, notably by Karl Meyer and his co-workers.

b) Nomenclature

The classification of proteinpolysaccharide complexes is confused by the indiscriminate use of such names as mucopolysaccharide, mucoprotein, mucosubstance, mucoid and mucin. The application of the systematic nomenclature to even the relatively simple constituents such as glucosamine (2-acetamido-2-deoxyglucose) appears to non-chemists to be unnecessary and cumbersome and the trivial names such as galactosamine and sialic acid are

preserved. The terms hyaluronic acid and heparin are used but the terms chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate are used to replace chondroitin sulphate A, C and B respectively, as proposed by Meyer (1959). The term chondroitin sulphate (CSA) is used where reference is to both isomers. No term has been proposed to supersede chondroitin sulphate D (the 'over sulphated' chondroitin 6-sulphate).

A classification of 'mucoid substances' based on the relative proportions of protein to polysaccharide (see Table I) has been proposed by Roseman (1959) but has failed to gain general acceptance and the prefix muco-, as recommended by Jeanloz (1960), is to be avoided and the term glycosaminoglycan to be used for polysaccharides containing amino sugars.

Table I

Carbohydrate %	Names	Examples
0	Protein	Insulin
Trace - 15	Glycoprotein Protein	Albumens Collagens
10 - 85	Mucoproteins	Orosomucoid Blood group substances
65 - 100	Mucopolysaccharides	Hyaluronic acid Chondroitin sulphate
100	Polysaccharides	Cellulose Glycogen

A classification of proteinpolysaccharide compounds, based on the ease with which the link between the protein and carbohydrate moieties can be split, is used by Schields (1963). Where there is a loose bond between protein and polysaccharide which can be broken easily, e.g. hydrogen bonds or salt linkages, the term mucoprotein is used. If the link is so stable that separation of the carbohydrate and protein can only be achieved by degradation

of the molecule into its individual carbohydrate and amino acid units, the term glycoprotein is used. To avoid the use of the term mucoprotein and to bring the classification into line with that used for protein-lipid complexes (lipoproteins and proteolipids), the use of the term proteoglycans for the relatively unstable protein-polysaccharide complexes is recommended. The term proteoglycan was recently coined (Balazs, quoted by Castellani et al., 1966), to cover all carbohydrate-protein complexes.

Proteinpolysaccharides

Stable bond	Unstable bond
Glycoproteins	Proteo-glycan
e.g. seromucoid, collagen	e.g. PP-L, hyaluronic acid

It is interesting to note that parallel to the nomenclature for the lipid-protein complexes, glycoproteins have predominantly the properties of proteins whereas proteo-glycans have properties very similar to those of glycosaminoglycans. In general, however, the classification used by Balazs and Jeanloz (1965) will be followed.

c) Isolation of Proteoglycans from Cartilage

As mentioned earlier, for many years following the isolation of a proteoglycan by Mörner (1889) and Schmiedeberg (1891) little work was carried out on these complexes. Hisamura (1938) claimed to have isolated a 'chondromucoid' from whale and bovine nasal cartilages but as the complex was made by precipitation at an acid pH it has been suggested (Schubert and Hamerman, 1965) that it was an artefact being the salt of a cationic protein and an anionic polysaccharide.* Partridge (1948) isolated a proteoglycan from nasal cartilage avoiding acid or alkaline conditions and using alcohol to deswell the collagen. He obtained a product containing 8 - 12% N which electrophoretically appeared to be a mixture. Shatton and Schubert (1954) improved this procedure in an attempt to produce a homogeneous product. They extracted the cartilage with a large volume of water, added two volumes of ethanol, which facilitated the removal of debris by centrifugation, and added potassium acetate to the extract.

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* It is interesting to note that the 'chondromucoid' of Hisamura contained two carbohydrate moieties: Hisamura recognised chondroitin sulphuric acid and another polysaccharide comprised of galactose and N-acetylglycosamine (identified as galactosamine).

This last step was a useful addition as the polyelectrolytic proteoglycan is only precipitated by alcohol in the presence of excess counter ions. This procedure was further improved when it was realised that rapid extraction was important to minimise the effect of degradative enzymes on the proteoglycan. Malawista and Schubert (1958) used thirty volumes of water in their extraction procedure, which was carried out in a high speed homogeniser (45,000 r.p.m.) at 0°C for only fifteen minutes. Subsequent isolation was by their previously reported method and it is claimed that the whole procedure takes only two hours. Other workers during this period were using much longer extraction times, for example, Mathews and Lozaityte (1958) used aqueous extraction of forty-eight hours and Bernardi (1957) used 30% aqueous potassium chloride for a similar period. Early studies on Schubert's proteoglycan suggested that it was a single compound; it resisted attempts at fractionation and was electrophoretically homogeneous up to pH 11 whence it underwent irreversible decomposition into the protein and polysaccharide moieties. (Warner and Schubert, 1958; Partridge and Elsden, 1961). However, when subjected to ultracentrifugation this 'chondromucoprotein' appeared to

consist of two components: a heavy fraction which rapidly sedimented and a slight fraction which comprised 75% of the complex. These are known as PP-H and PP-L respectively (Gerber, Franklin and Schubert, 1960). Although earlier work (Shatton and Schubert, 1954) had stated that the crude complex was free from collagen it undoubtedly does contain significant amounts (Pal and Schubert, 1965). The fraction referred to as PP-L is probably a purified form of the proteinpolysaccharide and contains less than 1% of collagen. The PP-H fraction contains 10-30% collagen (Pal and Schubert, 1965) and is probably a mixture of PP-L and collagen, some of it being specifically bound to the collagen fibres (Serafini-Fracassini and Smith, 1966).

It is the fraction known as PP-L, extracted from bovine nasal cartilage, upon which most of the studies embodied in this thesis were carried out. It is believed to represent the proteinpolysaccharide complex as it exists in the in vivo state (Serafini-Fracassini and Smith, 1966), and its composition and possible structures will be discussed later.

Using the method of Shatton and Schubert (1954), or minor modifications of it, proteoglycans have been found

in other tissues. PP-L has been isolated from both human and bovine articular and costal cartilage, though in smaller yields than from nasal cartilage (Schubert, 1965). Similar products have been isolated from chicken epiphyseal cartilage (Fitton Jackson, 1964), and from porcine metaphyseal cartilage (Castellani, 1965). Attempts to isolate an analogous product from bovine cornea were unsuccessful (Castellani, 1965).

The main differences between the proteoglycans isolated from the different cartilages are to be found in their sedimentation coefficients and protein content. Values for the S^{o}_{20w} range from 4.0s for preparations from nasal cartilage (Cessi and Bernardi, 1965) to 20s for preparations from epiphyseal cartilage (Fitton Jackson, 1964). Protein contents have been reported ranging from 7.5% for a highly purified preparation from nasal cartilage (Partridge, Whiting and Davis, 1965) to a value of 40% for a preparation from costal cartilage (Rosenberg, Johnstone and Schubert, 1965). In the case of the articular cartilage proteoglycan the ratio of protein to polysaccharide increases with age and with osteoarthritic changes (Bollet, Handy and Sturgill, 1963).

The wide variation in composition, particularly with respect to the protein content, indicates a lack of purity of the proteoglycan in spite of the electrophoretic and ultracentrifuge patterns showing a single peak. Some of the purification procedures that have been used will now be discussed.

d) Purification of the Proteoglycan, PP-L

The purification of a compound of high molecular weight which gives a highly viscous solution is difficult. Most of the methods employed are similar to those used in the purification and separation of individual acid mucopolysaccharides. Assessment of the purity of the complex is uncertain; the relatively crude material exhibits electrophoretic and ultracentrifuge homogeneity, criteria which are often taken to imply purity. It is also very difficult to be sure that none of the purification processes have depolymerised, degraded or denatured the molecules. Another of the difficulties is illustrated by the following example. The two isomers, chondroitin 4-sulphate and chondroitin 6-sulphate, isolated from bovine tracheal cartilage, may be differentiated by fractional precipitation of their calcium salts by ethanol. The former is

precipitated by 30-40% ethanol whereas the latter is precipitated by 40-50% ethanol. When this separation procedure is applied to a bovine nasal cartilage preparation of chondroitin sulphate, two fractions are again obtained but one of the isomers, chondroitin 6-sulphate, has not been demonstrated in this tissue (Meyer, Davidson, Linker and Hoffman, 1956).

Ultracentrifugation (78,000 g. for two hours), as introduced by Gerber, Franklin and Schubert (1960) was an important step in the purification of the proteoglycan. Partridge, Whiting and Davis (1965) have attempted to carry the purification procedure further. They noted the variable protein content and found that attempts to purify the material by precipitation from aqueous solutions with ethanol led to a fall in its protein content. Fitton Jackson (1964) interpreted her negatively stained preparation of PP-L, viewed by electron microscopy, as indicating a central fibrous protein core surrounded by doughnut-shaped rings of a globular protein. Since this loosely associated globular protein was not in keeping with the model structure previously proposed for the proteoglycan (Partridge and Davis, 1958), attempts were made to

fractionate the material by gentle physical means aimed at removing all the protein not covalently combined to the polysaccharide, i.e. the globular protein referred to above. First of all, however, the autodegradation of the PP-L fraction had to be overcome. Partridge, Whiting and Davis (1965) showed that a solution of the proteoglycan maintained at 37°C showed an irreversible fall in viscosity which was accompanied by the liberation of carboxyl groups: after exhaustive autodigestion, amino acid and small peptides could be isolated. Malawista and Schubert (1958) had noted a fall in viscosity of incubated solutions of the proteoglycan but stated that if the solution was taken to dryness and then redissolved, the viscosity returned to the initial level. This could be interpreted as a reversible change in the molecular configuration, or orientation within the solution, rather than degradation. Addition of 6mM DFP was shown to completely inhibit the degradation noted by Partridge, Whiting and Davis (1965). The material was fractionated on a long column of DEAE cellulose using a potassium chloride gradient at 0°C and was found to give two main fractions. The fore peak

consisted entirely of protein and was considered to be the non-covalently linked globular protein. The main peak was considered to be the purified proteoglycan; it contained 7.5% protein and had a higher viscosity than purified chondroitin sulphate. However, on ultracentrifugal analysis it was shown to consist of two components with S°_{20w} values of 0.95 and 2.3. Osmotic studies of the molecular weight of this proteoglycan indicated it to be less than 750,000 (the value proposed for the complex by previous studies [Partridge and Davis, 1958]). The isolated protein had a similar amino acid composition to the unpurified PP-L. Further studies are necessary to establish the relationship between this highly purified product and the in vivo state. Muir (1958) purified the proteoglycan, which was isolated from pig hyaline cartilage by precipitation from aqueous solutions of the 5-aminoacridine salt, to give a product with a protein content of 10%.

Partridge and Davis (1958) used an ion exchange resin (Amberlite C. G. 50) to purify their crude proteoglycan and found that it was particularly effective in removing the contaminating collagen.

The recently introduced techniques of separating acidic polysaccharides by means of fractionating their complexes with cetyl pyridinium chloride has been applied to the purification of proteoglycans (Partridge, Whiting and Davis, 1965), papain digestion products of cartilage (Kent and Stevenson, 1965) and to a protein-chondroitin 6-sulphate complex isolated from aorta (Buddecke and Schubert, 1961).

When a polyanion is mixed with an aqueous solution of cetyl pyridinium chloride (CPC) an insoluble complex is formed. Above a certain temperature, the 'critical solution temperature' (CST), polyanions with different charge densities may be distinguished by their different solubilities in salt solutions. This solubility is constant and characteristic for each polyanion and is referred to as the 'critical salt concentration' (CSC), (Scott, 1960). For example, if the CPC complexes of hyaluronic acid, chondroitin sulphuric acid and heparin are extracted with aqueous magnesium chloride, they dissolve at concentrations of 0.6, 2.0 and 3.0 Molar respectively, (Antonopoulos, et al., 1961). Few details have been given of the application of this technique to the purification of protein- or peptide-polysaccharide complexes. Partridge, Whiting and Davis

(1965) used it to remove degradation products of the DEAE cellulose column from their purified PP-L. Kent and Stevenson (1965) applied it unsuccessfully to the separation of chondroitin sulphate from chondroitin sulphate and keratan sulphate peptide complexes. Buddecke and Schubert (1961) used essentially the same technique, as will be described later in the experimental section.

e) Other Proteoglycans

When it was realised that most, if not all, of the chondroitin sulphate of nasal cartilage was bound to protein (Campo and Dziewiatkowski, 1962) other protein-polysaccharide complexes were sought. Although more work has probably been done on the proteoglycan known as PP-L, brief mention must be made to these other complexes.

Glycoproteins such as ovalbumin and α_1 globulin contain carbohydrate and protein moieties but polymers containing either, exclusively, cannot be isolated from them. Ogston and Stanier (1950) first described a 'complex' of hyaluronic and protein which appears to contain about 30% protein. It migrated as a single peak in an electrical or gravitational field and had a

higher particle weight than the isolated protein-free polysaccharide, but it appeared to be an association of protein and polysaccharide rather than a definite compound (Rogers, 1961). For example, Curtain (1955) has shown that the protein exchanges with other proteins in the surrounding fluid and it may be that the molecules are in some way electrostatically trapped in the interstices of the skein of hyaluronate.

The evidence for the existence of proteoglycans containing other glycosaminoglycans is not well established and is indirect. Extraction of other glycosaminoglycans from tissues is facilitated by alkaline treatments and proteolysis, in the same way that chondroitin 4-sulphate is released from PP-L. Studies on the nature of the proteinpolysaccharide linkage, to be considered, later indicate the role of serine in these linkages. Thus, the presence of serine as the principal amino acid residue in various preparations of heparin is evidence of the existence of a heparin-protein complex, in spite of the fact that direct evidence is rather equivocal (Lindahl, Cifonelli, Lindahl and Roden, 1965). Similarly, there is evidence that chondroitin, dermatan sulphate and corneal keratan

sulphate form asparaginyl-glycosyl links to protein (Meyer quoted by LLoyd, 1965).

f) Structure and Properties of the Proteoglycan, PP-L

Soon after the isolation of this complex it was found that the main constituents were a non-collagenous protein and chondroitin sulphuric acid. The earlier, cruder preparations had CSA and protein contents of 60-75% and 25-40% respectively (Bernardi, 1957) but the composition of the purified PP-L fraction was approximately 15% protein and 85% polysaccharide (Gerber, Franklin and Schubert, 1960). The I. R. spectrum indicates that the polysaccharide is predominantly chondroitin 4-sulphate (Campo and Dziewiatkowski, 1962). The amino acid composition of the protein has been reported (Scheinthal and Schubert, 1963), and indicates a completely different composition from elastin or collagen. There are approximately twice as many acidic amino acids as basic amino acids in the protein. Partridge and Elsden (1961) isolated the protein from the complex by alkaline treatment and showed that it could be incompletely separated into two fractions by ion exchange chromatography. Both fractions, however, had a similar amino acid composition

and it was concluded that they were both derived from the protein moiety which had been degraded by the action of alkali. Analysis of the complex showed that in addition to the galactosamine derived from the chondroitin 4-sulphate, glucosamine comprised approximately 14% of the total aminoglycans (Partridge, Davis and Adair, 1961). The glucosamine was found mainly in the protein isolated by alkali denaturation of the complex. This finding, together with that of considerable amounts of galactose in the protein moiety, led to the suggestion (Partridge and Elsden, 1961) that keratan sulphate forms the major alkali-stable carbohydrate in the protein core, together with small amounts of galactosamine and uronic acid which are the residue of the chondroitin 4-sulphate split off by the alkali. Keratan sulphate was isolated from PP-L by Gregory and Roden (1961). It has also been shown to contain approximately 1% sialic acid (Anderson, 1961; Pal and Schubert, 1965). The role of the sialic acid is not clear; it may be a contaminant. Fractionation studies of the alkaline degradation products of PP-L (Scheinthal and Schubert, 1963) indicate that it is mainly associated with the protein-keratan sulphate moiety. This is in

agreement with the statement that uronic acids and sialic acids are mutually exclusive from acidic polysaccharides (Gottschalk, 1955). The presence of galactose and xylose in glycopeptides isolated from the complex (Gregory, Laurent and Roden, 1964) will be considered in the section on the nature of the protein-polysaccharide linkage. Thus, the known components of the proteoglycan, PP-L, are 15% protein and keratan sulphate, which comprises 15% of the polysaccharide moiety, forming an alkali-stable bond with the protein. The other polysaccharide component is chondroitin 4-sulphate, although it has not been possible to exclude a chondroitin 6-sulphate content of less than 10% (Scheinthal and Schubert, 1963), together with traces of xylose, galactose and sialic acid associated with the protein moiety.

Studies were next directed at finding out the size and shape of the molecule and finding out the arrangement of the individual components within the molecule. From light scattering data Bernardi (1957) estimated the molecular weight to be 1.98×10^6 whilst Mathews and Lozaityte (1958) reported values of $2 - 5 \times 10^6$. Webber and Bayley (1956) gave a value of 1×10^6 for the molecular weight of the complex

estimated by sedimentation and viscosity measurements. These workers also estimated the average molecular weight of the chondroitin 4-sulphate chains after their release from the complex with trypsin. They reported a value of 39,000 by sedimentation and viscosity measurements but a value of 145,000 from light scattering studies. Mathews, however, found a value of 50,000 for the average molecular weight of the chondroitin 4-sulphate chains and his proposed model for the complex (Mathews, 1965) is as follows:- A protein core 4,000 $\mathring{A}$ long to which are covalently bound about sixty chondroitin 4-sulphate chains of molecular weight 50,000 and contour length approximately 1,000 $\mathring{A}$.

Partridge, Davis and Adair (1961) estimated the weight of the chondroitin 4-sulphate chains by comparing the reducing power of the terminal residue with the osmotic molecular weight of the complex. They performed these measurements before and after treatment with papain, and with hyaluronidase. Their proposed model is smaller than that of Mathews and is as follows:- A macromolecule of molecular weight $1 - 5 \times 10^6$; probably an aggregate of units having a molecular weight of 750×10^3. This consists of a protein core of molecular weight 120,000

associated with keratan sulphate. Attached to the core are approximately twenty-three chondroitin 4-sulphate chains of weight 28 x 10^3. *

Early studies on the molecular form of the proteoglycan were performed by Webber and Bayley (1956). They used a rather crude preparation which had probably been degraded but attempted to remove any free protein with phosphotungstic acid. They concluded, as a result of molecular weight determinations, before and after tryptic digestion, that the polysaccharide chains were linked end to end by polypeptides. They demonstrated streaming birefringence and concluded that the molecule was 4,000 Å long and that it was a macromolecule built up from highly charged, flexible coils. Mathews and Lozaityte (1958) proposed a basically rod-like structure which could form macromolecules by aggregating both laterally and end to end, the aggregates linking by hydrogen bonds giving structures of molecular weight up to fifty million.

Bernardi (1957) came to similar conclusions as

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* Recently Partridge (1966) has proposed an even smaller molecular model for PP-L. It has a molecular weight of 250,000 and consists of 8-9 chondroitin sulphate chains attached to a peptide core of weight 16,000 and is linked covalently to a globular protein of diameter 60 Å.

Webber and Bayley (1956), although he was unable to confirm the streaming birefringence. Cessi and Bernardi (1965) performed definitive experiments to decide which of the two proposed models for the proteoglycan was valid. On one hand there was the model of Mathews and Lozaityte (1958), and Partridge, Davis and Adair (1961), which consisted of a single protein core attached to which were chains of chondroitin 4-sulphate. On the other hand, Bernardi (1957), and Webber and Bayley (1956), postulated a model which consisted of chondroitin 4-sulphate chains linked end to end by short polypeptides. They measured the molecular weight by viscosity, sedimentation and light scattering data of the complex during enzymic digestion with papain and with hyaluronidase. The papain caused a much more marked and complete fall in molecular weight than hyaluronidase and this result, they concluded, was unequivocally in favour of the models of Mathews and Partridge.

They followed the enzymic degradation with papain, by estimating the terminal $-NH_2$ groups with FDNB, using both spectrophotometric and radiochemical techniques. The undegraded proteoglycan had approximately one hundred terminal NH_2 groups per molecule; they record

the molecular weight as 2.2×10^6. This is a high figure but can probably be accounted for by the fact that their complex is somewhat degraded; they give a sedimentation coefficient of 4.0s. This is the only reference to the application of the fluorodinitrobenzene technique for the determination of N terminal groups, to the proteoglycan PP-L, apart from a failure to find-NH_2 terminal amino acids in hyaluronidase treated PP-L (Sjöquist quoted by Gregory, Laurent and Rŏden, 1964).

Fitton Jackson (1964) has proposed a different macromolecular arrangement for her 20s proteoglycan. She negatively stained her preparation with phosphotungstic acid and measured the size of the molecule on the electron microscope. The pictures indicated an elliptical sub-unit 55 Å x 45 Å. Many of the sub-units were arranged in rings, made up of five or six sub-units, which measured 165 Å x 45 Å or 165 Å x 55 Å. The molecular weight from sedimentation and viscosity measurements was 1.16×10^6. Attached to the ring of protein sub-units were chondroitin sulphate chains, 2-4 chains per sub-unit, giving a total diameter to the complex of approximately 220 Å.

Examination of the residue from the extracted chick epiphyseal cartilage by the electron microscope indicated numerous filaments, 200 - 300 Å in diameter. These filaments were shown to be distinct from the occasional collagen fibres. It was considered from the results of enzyme studies that the filaments were built up by the piling of one on top of another of the aggregates seen in the 20s fraction. An alternative macromolecular arrangement suggested was that five sub-units basically form linear aggregates which in the aqueous extract gave rise to ring forms, but in the natural state associated laterally or helically, to form the filament. The filament, however, may be built up of direct aggregation of the basic sub-units. It is postulated (Fitton Jackson, 1964) that there may be more than one protein present in the complex; this is supported by the work of Partridge, Davis and Whiting (1965), which indicates that not all of the protein is covalently bound to the carbohydrate moieties. It is postulated (Fitton Jackson, 1965) that each acid polysaccharide in a tissue is attached to its own specific protein rather than all to the same non-collagenous protein.

Some of the important properties which this proteoglycan exhibits both *in vivo* and *in vitro* are dependent on the fact that it behaves as a polyelectrolyte. Because of this, and as some of the experiments to be described later depend on this phenomenon, PP-L will be briefly considered in this light. The term, polyelectrolyte, was proposed by Fuoss for a group of substances which are polymers but which behave in aqueous solutions as electrolytes (Fuoss and Strauss, 1948). The chondroitin 4-sulphate chains, of the proteoglycan PP-L, act as polyanions by virtue of their ionisable carboxylic and sulphate groups. Surrounding these anionic groups will be an atmosphere of small ions comprising mainly counterions together with some coions of low molecular weight salts. *In vivo*, the main counterions would be Na^+ with some K^+, Ca^{++} and Mg^+ ions present in smaller quantities. The main coion is Cl^-, with HCO_3^- and PO_4^{---} ions forming a sizeable minority. The polyanionic polysaccharides behave as weak electrolytes, i.e. they behave as though a considerable fraction of the cationic counter ions were not altogether free. In the case of chondroitin sulphate, 50% of Na^+ or K^+ ions, 70% of Ca^{++} ions and

an even higher percentage of trivalent counter ions behave as though they were bound (Schubert, 1964). These polyvalent cations will precipitate acid polysaccharides from aqueous solutions, but they can be displaced from their sites of attachment by univalent counterions provided that there are enough of these counterions in the immediate environment. This phenomenon is utilised in one of the purification procedures for PP-L, to be described later.

An uncharged linear polymer in aqueous solution will exist in a randomly kinked form provided that there are no significant intra-molecular forces. In a polyanionic chain, two opposing factors will be introduced. Firstly, numerous hydrogen bonds will exist between the carboxylic groups tending to contract the chain. Secondly, any ionised carboxylic and sulphate groups will strongly repulse each other and tend to stretch the chain, (Katchalsky, 1964). In the case of chondroitin 4-sulphate chains, this second factor will predominate and they will normally exist as extended chains attached to the protein core. Addition of acid to a solution of a polyanion will tend to collapse the chains by virtue of the suppressed ionisation of the

carboxylic and sulphate groups. Addition of acetone to the solution will have two opposing effects. Firstly, it will suppress the ionisation of the carboxylic groups, (Davis and Smith, 1955), tending to collapse the chain. Secondly, by lowering the dielectric constant of the medium, it will increase the repulsive forces between the charged groups. It is suggested that with a polyanion-like chondroitin 4-sulphate which has many divalent sulphate groups, the latter will be the major effect of acetone. From what has been said previously, it would be expected that the addition of a trivalent counterion such as Bi^{+++} to an aqueous solution of the proteoglycan will cause the chains of chondroitin 4-sulphate to collapse and adopt a coiled configuration. It is also suggested that the trivalent Bi^{+++} would 'cross link' between SO_4^{--} groups of adjacent chondroitin 4-sulphate chains. The use of bismuth nitrate, in aqueous and in acetone solutions of nitric acid, as an electron microscope stain and as a means of purifying PP-L is mentioned in the experimental section.

The polyanion attributes are responsible for many other properties of the proteoglycan PP-L. Some of the more interesting will be mentioned. Polyanions and polycations will interact to form a coacervate; the

interaction of nucleotides with basic proteins to form nucleoproteins is a similar phenomenon (Felenfeld and Rich, 1957). A further example is provided by Bloom and Blake (1948). They showed that the organism B. anthracis releases large amounts of polyglutamic acid when it invades mammalian tissues. This exotoxin acts as a spreading factor opening up channels between cells and facilitating the penetration by the bacillus. The host produces a polycation tissue factor which combines with and neutralises the exotoxin. Certain stains which are used to visualise the polyanionic polysaccharides are polycations and their interaction fixes the stain, enabling the polysaccharide to be visualised. Alcian and Toludine Blue act in this manner. Cetyl pyridinium chloride, in aqueous solution forms micelles, which act as highly charged polyvalent cations, and can react with polyanions to give precipitates. This is the basis of the use of this compound in the separation and isolation of different polyanions.

Katchalsky (1964) has shown how polylysine can firmly 'glue' erythrocytes together and has shown that these molecules are orientated at the surface of cells and fibres. These orientated polyelectrolytes would be

expected to alter the phase of polarised light passing through them and Vidal (1964) showed that hyaluronidase decreased the form birefringence of collagen fibres in sections of tendon. The interaction of polyanions with cell surfaces and with collagen fibres undoubtedly is of major importance in the organisation of tissues at the microscopic level (Mathews, 1965).

An interesting phenomenon, which may be of importance in the question of associated impurities in PP-L, arose during an attempt to measure the excluded volume in a sample of PP-L. Ogston and Phelps (1961) used a Millipore chamber to investigate the effect of hyaluronic acid on the equilibrium of albumen across a filter which was permeable to albumen but not to the hyaluronate. They found that when equilibrium was established the hyaluronate solution acted as though part of its volume was unavailable to the albumen, i.e. it tended to exclude the albumen from part of its micro environment. When the same experiment was performed using a sample of PP-L instead of hyaluronate at low salt concentrations the excluded volume effect was completely abolished and the system behaved as though the albumen were bound by the PP-L (Schubert, 1965).

This affinity of proteins for the domain of the proteoglycan PP-L means that purification will be difficult as the use of salt solutions is essential for many of the purification procedures used. Aqueous solutions of PP-L and solutions in salts greater than 0.2M, however, behave in a similar manner to hyaluronate in the Ogston and Phelps chamber (Gerber and Schubert, 1964).

Recently, the antigenic properties of this proteoglycan have been investigated. Antibodies to porcine thyroid and cricoid cartilagenous proteoglycans were demonstrated in rabbit's serum after three injections of the proteoglycan, emulsified with Freund's complete adjuvant, (Loewi and Muir, 1965). These workers detected a variety of antibodies presumably directed at different fractions of the proteoglycan; the majority of these were only revealed after hyaluronidase treatment. The antibodies were found mainly in the γ_2 globulins but some were undoubtedly macroglobulins and γ_1 globulins. Using an immuno-fluorescent technique these workers attempted to localise the proteoglycan in sections of cartilage. Barland, Janis and Sandson (1966) extended the immuno-fluorescent technique to the study of osteoarthritic cartilage. They found that increased fluorescent

staining occurred in the superficial layers of the osteoarthritic cartilage which paralleled the loss of metachromasia. The immuno-fluorescent technique like the histochemical stain for kerato-sulphate (Stockwell and Scott, 1965), only stains the immediate pericellular zone and not the main mass of the cartilage matrix which undoubtedly contains the proteoglycan, but which is specifically attached to the collagen fibre and is presumably unavailable to the labelled antibody (Smith, Peters and Serafini-Fracassini, 1966). Pretreatment of the tissue sections with hyaluronidase (Loewi, 1965), and with papain and lysosomal enzymes for short periods, increased the immuno-fluorescent staining producing an almost identical appearance to that found in osteoarthritis. Prolonged predigestion of the tissue section with papain, abolished the immuno-fluorescent staining capacity. Loewi and Muir (1964), like previous workers, were unable to demonstrate antibodies against chondroitin sulphate, hyaluronic acid and kerato-sulphate. Barland, Janis and Sandson (1966) showed that the antibodies were directed against the protein rather than the carbohydrate moieties of the proteoglycan. Di Ferrante and Pauling (1965), however,

claim to have demonstrated precipitating antibodies directed at the carbohydrate moiety as well as antibodies directed against the protein component. The immuno-fluorescent technique is a valuable addition to the histologists armamentarium for the investigation of cartilaginous tissue and the immuno-electrophoretic studies of Loewi and Muir (1964) indicate the heterogeneity of the proteoglycan and should prove a useful technique for assessing the purity of other complexes.

Finally, in this section on the structure and properties of the proteoglycan, mention must be made of one of the interesting phenomenon found by Schubert and his co-workers. The chondroitin 4-sulphate chains may be separated from the rest of the compound by either treatment with alkali or by treatment with trypsin. Using the method of Partridge, Davis and Adair (1961), Scheinthal and Schubert (1963) showed that the chain weight of the trypsin-released chains approximated to that found in the original proteoglycan but that chains released by the action of alkali had a chain weight approximately half that of the trypsin-released chains. This has recently been confirmed by Doganges and Schubert (1964), using the unusual properties of the lanthanum ion

to effect a separation of the chondroitin 4-sulphate chains from any undegraded proteoglycan. At 4°C the proteoglycan PP-L is reversibly precipitated from aqueous solution by the La^{+} ion whereas the chondroitin 4-sulphate chains are only precipitated at 50°C. No adequate explanation has been suggested for this phenomenon but it is hoped to consider the matter further in the section on the proteinpolysaccharide linkage.

g) The Nature of the Proteinpolysaccharide Linkage

Considerable interest has been shown recently in this aspect of proteoglycans, particularly as it has been suggested (Knecht and Dorfman, 1965) that a failure to synthesise this linkage is the 'metabolic lesion' in gargoylism (Hurler's Syndrome).

Ever since Krukenberg (1884) and Schmiedeberg (1891), showed that alkaline extracting media were more effective in extracting glycosaminoglycans from tissues than neutral or acidic solutions, a salt or ester link between the two moieties has been assumed. Levene (1925) concluded that there was an ester link between the protein and polysaccharide, (particularly chondroitin sulphate), components of proteoglycans. Following the isolation

of a proteoglycan in large yields by Schubert and his co-workers, more intensive studies on the nature of the proteinpolysaccharide linkage, particularly the alkali labile protein-chondroitin sulphate link, were possible.

Blumenfeld and Gallop (1962) have shown that treatment of collagen with 1M. hydroxylamine or hydrazine at pH 9 - 10 split each tropocollagen sub-unit into six components and showed (Blumenfeld, Rojkind and Gallop, 1965) that these reagents split ester linkages between adjacent aspartic acid residues in the polypeptide chains. These reagents have been applied to the problem of the proteinpolysaccharide linkage in PP-I by three groups of workers; Andersen, Hoffman and Meyer (1963); Gregory, Laurent and Roden (1964), and Pal and Schubert (1965). They were unable to demonstrate significant hydrazide in the reaction products and Pal and Schubert (1965) were unable to isolate any chondroitin 4-sulphate chains using La^{+} ions. Thus it is unlikely that the proteinpolysaccharide bond is an ester linkage. It is interesting to note, however, that Pal and Schubert (1965) suggest that the proteoglycan is itself bound in tissues by ester links.

An important advance in the elucidation of the nature of the proteinpolysaccharide linkage was made by Muir (1958). She found that serine was the principal amino acid in a sample of pig tracheal proteoglycan exhaustively treated with papain, and she suggested that the polysaccharide chains were linked to the polypeptide by an alkaline labile linkage through serine. Andersen, Hoffman and Meyer (1963) used an elegant technique to demonstrate the role of serine and threonine in the proteinpolysaccharide linkage. The amount of serine and threonine was determined in the proteoglycan before and after alkali treatment (48 hours, 0.5 N NaOH at 25°C). Any serine or threonine involved in O glycosidic linkages through their hydroxyl groups would be preferentially destroyed by the alkali. When applied to the proteoglycan PP-L only serine was found to be implicated. Application of this technique to other proteoglycans, for example those found in Morquio's disease (Eccentro-osteochondrodysplasia), human tracheal cartilage, blood group substances and sub-maxillary glands, indicated that both threonine and serine were implicated (Adams, 1965; Castellani, Zonta, Balduni

and Laterza, 1966). This latter group of workers has also confirmed the role of serine as the sole linking amino acid in the proteoglycan, PP-L.

Further studies were made on the glycopeptides isolated from PP-L which had been exhaustively digested with papain and hyaluronidase. The glycopeptides were fractionated on Sephadex and digested further with pronase and leucine aminopeptidase. Serine was demonstrated as the sole amino acid but an unexpected finding was of galactose and xylose.

Isolation of several fragments from this region including glucuronosyl-galactose, xylosyl-serine, galactosyl-xylosyl-serine, galactosyl-galactosyl-xylose and the terminal trisaccharide, glucuronic acid-N acetyl galactosamine-glucuronic acid, from the chondroitin 4-sulphate chain have enabled a fairly clear picture of the nature of the linkage region to be built up (Roden, 1965; Roden and Lindahl, 1965; Roden and Armand, 1966). Serine is linked O glycosidically to the reducing carbon of xylose. The xylose is linked to galactose by a β 1→3 glycosidic bond, which is linked to a further galactose unit. The nature of these linkages is not clear but all the glycopeptides produced were non-reducing. The second galactose

is linked to glucuronic acid by a 1→3 β glycosidic bond. The O glycosidic link between serine and xylose would be cleaved by alkali, yielding a reducing end group. This could explain the observation of Scheinthal and Schubert (1963) that the chain weight of the alkali-released chondroitin 4-sulphate was twice that of the trypsin-released chains which would still contain the terminal amino acids. The fact that the chondroitin 4-sulphate chain was attached to the polypeptide by its reducing end meant that the estimation of chain weight by the reducing method of Partridge, Davis and Adair (1961) must be interpreted with caution. The chains are unbranched and attached at only one site on the protein core (Gregory, Laurent and Roden, 1964). Consequently, the free end of the chain would be non-reducing. Disparity of results for the chain weight in the native proteoglycan and in the papain-treated product, and disparity between different groups of workers, together with the recent finding (Beeley and Jevons, 1963) that certain protein components can give apparent reducing groups, is further evidence of the unreliability of this technique.

Little is known of the nature of the keratan

sulphate-protein linkage in PP-L apart from the fact that it is an alkali-stable bond. It is proposed to consider some other proteinpolysaccharide bonds as far as they have been elucidated. Neuberger and his colleagues have extensively studied the carbohydrate-protein linkage in ovalbumin and have shown that it is an N-(β-aspartyl) glycosylamine linkage (Marks, Marshall and Neuberger, 1963). This N-glycosidic link would be expected to be alkali-stable (Salton, 1965). There is evidence that this type of linkage occurs in the keratan sulphate-protein complex which exists in bovine cornea. After exhaustive proteolysis an alkaline-stable keratan sulphate-peptide was isolated which was found to have aspartic acid as the predominating amino acid (Castellani, 1965). This linkage is not the same as the alkaline-labile bond implicated by Murphy and Gottschalk (1961) in bovine submaxillary mucin (B.S.M.). The majority of the protein-carbohydrate linkages are glycosidic ester links between the C-1 hydroxyl groups of the sugar moiety and either the β-carboxyl group of aspartic acid of the γ-carboxyl group of glutamic acid. The remaining linkages in B.S.M. are O-glycosidic bonds involving

serine and threonine. Meyer quoted by Lloyd (1965) considers that chondroitin, dermatan sulphate, as well as corneal keratan sulphate form N-(β-aspartyl) linkages with their respective proteins. It does not appear, as was once thought, that each polysaccharide forms only one type of link with protein. Thus keratan sulphate forms O-glycosidic links with serine and threonine as well as N-(β-aspartyl) linkages. Keratan sulphates isolated from different sources differ in their associated amino acids. Thus a preparation from bovine cornea has predominantly aspartic acid residues whilst that from human costal cartilage is rich in proline (Meyer, Seno, Anderson, Lipman and Hoffman, 1964). This group of workers have also shown corneal keratan sulphate is associated with chondroitin 4-sulphate whilst costal cartilaginous keratan sulphate is associated with chondroitin 6-sulphate, in their respective protein complexes. There is evidence that dermatan sulphate can form O-glycosidic links with serine as well as the N-(β-aspartyl) links, suggested by Meyer. Roden (1965) has shown that serine is the principal amino acid residue in isolated dermatan sulphate. Galactose, xylose and glucuronosyl galactose have been found in hydrolysates

of this polysaccharide; the finding of glucuronic acid, in view of the fact that L-idiuronic acid is the main uronic acid component of dermatan sulphate, is particularly interesting. Heparin appears to be linked to protein by a similar galactose-galactose-xylose-serine bridge as found between chondroitin 4-sulphate and protein in the proteoglycan PP-L (Lindahl and Roden, 1965). Heparin sulphate-protein complexes, isolated from human aorta, are probably linked in a similar manner (Jacobs and Muir, 1963).

Thus there is evidence that the keratan sulphate in PP-L is linked O-glycosidically to both serine and threonine and possibly N-glycosidically to glutamic and aspartic acid (Meyer, Anderson, Seno and Hoffman, 1965). However, we may expect evidence of other links involving sialic acid and methyl pentose known to be intimately associated with keratan sulphate glycopeptides. Similarly, the intimate association of keratan sulphate with chondroitin 4-sulphate and with chondroitin 6-sulphate as doublets linked by protease resistant peptides, postulated by Meyer et al (1965) should be elucidated in the not too distant future.

h) Metabolism of cartilage proteoglycans

There is little doubt that chondrocytes are responsible for the synthesis and catabolism of proteoglycans. Using ^{35}S sulphate, various workers have demonstrated the synthesis of chondroitin sulphates, by chondrocytes, in a variety of different tissues, e.g. articular cartilage (Carlson, 1957), costal cartilage (Dziewiatkowski, 1962), epiphyseal cartilage (Guri, Slater and Bernstein, 1965) and tracheal cartilage (Carlson, 1957). Autoradiographic studies by these workers have indicated an intracellular synthesis and have shown that labelled sulphate is largely incorporated in the isolated chondroitin sulphate or into the chondroitin sulphate-protein complex. 3% of the labelled sulphate was found to be incorporated into taurine and only trace amounts into cystine and methionine (Carlson, 1957). Tissue culture experiments have shown that many mesenchymal cells can synthesise hyaluronic acid and chondroitin sulphate (Grossfield, Meyer and Godman, 1955). In an electron microscope study of chondrogenesis in rat epiphyseal cartilage, Godman and Porter (1960) have demonstrated that dense amorphous material,

presumably proteoglycans, was synthesised in the Golgi apparatus and that vesicles of this material make their way to the cell surface and discharge their contents into the pericellular zone.

An interesting study on the synthesis and catabolism of both parts of the proteinpolysaccharide complex of rat costal cartilage has been made by Gross, Mathews and Dorfman (1960). They injected rats with ^{35}S sulphate and ^{14}C lysine and isolated the protein-chondroitin sulphate complex at various times after the injections. Simultaneous labelling of the protein and carbohydrate moieties with ^{14}C and ^{35}S respectively was demonstrated, being maximal twenty-four hours after the injections. Both moieties were found to be catabolised at a parallel rate with a biological half life of approximately eight days. However, there was evidence that there was not a single metabolic pool. The catabolism of the proteoglycan did not demonstrate first order kinetics and the rate of synthesis of the alkali-extracted chondroitin 4-sulphate was less than that extracted in the proteoglycan. The half life of this former chondroitin 4-sulphate is significantly longer than that of the latter.

These findings have been confirmed using bovine costal cartilage slices incorporating ^{35}S sulphate into the chondroitin sulphate and ^{14}C leucine and ^{14}C phenylalanine into the protein moiety, by Campo and Dziewiatkowski (1962). This parallel synthesis and breakdown of both moieties of the proteoglycan is very significant evidence for the belief that PP-L represents a true unit both *in vivo* and *in vitro*. It is not clear what is the size of the sub-unit which is actually synthesised or how large is the macromolecule which is extruded by the cell into the inter-cellular matrix.

Application of the autoradiographic technique to the study of problems in pathology has already had some success. Carlson (1957) studied the process of repair of articular cartilage following injury, and demonstrated that the intermediate cell layer had the greatest potential for cell division and repair. Collins and McElligott (1960) investigated the synthesis of chondroitin sulphate by chondrocytes in both normal and osteoarthritic cartilage. They showed that not only was there no fall in the ^{35}S sulphate incorporation by chondrocytes with age, but that in osteoarthritic cartilage the chondrocytes demonstrated enhanced activity. This

latter finding is particularly interesting as it had been assumed that in osteoarthritis there was a loss of inter-fibrillary ground substance which caused a decrease in resilience of the articular cartilage leading to its destruction (Kellgren, 1965). This decrease in aminoglycans, first demonstrated quantitatively by Mathews (1953), was assumed to be due to impaired chondrocyte function but in view of the findings of Collins and McElligott (1960), it is suggested that there is a failure of binding of the proteoglycan to the collagen fibres or indeed a change in the integrity of the proteoglycan itself. Urgent studies are needed into the nature of this disease which has been shown to afflict over half the adult population (Lawrence, Bremner and Bies, 1966).

Campo (1964) has studied the fate of ^{35}S sulphate and ^{14}C leucine, dual labelled, metaphyseal cartilage during calcification and has shown that there is a progressive loss of the ^{14}C labelled material (protein) which was almost completely absent from the calcified matrix. The ^{35}S labelled material (chondroitin sulphate) persisted in the tissue throughout, although it was present in a reduced amount in the calcified matrix.

This dissociated catabolism of the protein and polysaccharide moieties is particularly interesting and indicates possible modes of the breakdown of proteoglycans. An enzyme has been partially purified from various cartilages which degrades PP-L, liberating the chondroitin 4-sulphate chains from the proteoglycan (Tourtellotte, Campo and Dziewiatkowski, 1963). These chains would be immediately excluded from the domain of the proteoglycan, as indicated by Schubert (1964), and further metabolised. Little is known of the further catabolism of chondroitin 4-sulphate but it is known that desulphation occurs as ^{35}S chondroitin sulphate and ^{35}S heparin give rise to ^{35}S inorganic sulphate in the urine (Danishefsky and Eiber, 1959) and the rest of the chain is presumably degraded by one or more 'mucopolysaccharidases' (Walker, 1961).

The action of proteolytic enzymes on the breakdown of the cartilage matrix has been studied fairly intensively over the past decade and the significance of these experiments with respect to the normal in vivo catabolism of proteoglycans will be considered. Thomas, in 1956, showed that an intravenous injection of papain led to the collapse of rabbit ears within a few hours, and

recovery within a few days. This phenomenon, which can also be produced by intravenous ficin or bromelin, leads to the decrease in tissue chondroitin sulphate and its simultaneous appearance in the blood and urine (McCluskey and Thomas, 1958; Bryant, Leder and Stetten, 1958). Proteolysis of the non-collagenous protein in the proteoglycan led to the release of the chondroitin sulphate which was excluded from the tissue.

Studies on the mode of toxicity of vitamin A has implicated the role of lysosomes in the catabolism of cartilage. Dr. Fell and her co-workers have shown that vitamin A releases cathepsins from the chondrocyte lysosomes which degrade the cartilage matrix (Dingle, 1964). It appears highly likely that several of the lysosomal enzymes play a part in the catabolism of proteoglycans including cathepsin I and II, desulphatase, β-glucuronidase and β-galactosidase. This extracellular degradation normally proceeds at an ordered rate but it has been suggested that in various pathological conditions increased numbers and probably increased fragility of the lysosomes play a part in the pathogenesis of disease, particularly of connective tissue disorders (Dingle, 1962). Thus it was shown that addition of

anti-serum to fibroblast tissue cultures led to the activation of lysosomes and that at 37°C release of the enzymes occurred within two to three minutes (Weiss and Dingle, 1964). This is in keeping with the suggested auto-immune nature of the so-called 'collagen diseases', particularly rheumatoid arthritis. Recently, the synovial tissue in this condition has been shown to contain a greatly increased number of lysosomes (Hammerman, Stephens and Barland, 1963) and it is interesting to note that hydrocortisone, which has a remarkably beneficial effect on the symptomatology of this disease, protects the lysosomes from rupture by a variety of agents including vitamin A, ultra violet irradiation and antisera (Dingle, 1964). Weissman (1964) has even suggested that the lysosomal proteases may themselves degrade normal tissue components to yield auto-antigenic moieties.

It is readily apparent from this brief review of proteoglycan metabolism that there are still many unsolved problems, most of which appear to have a direct bearing on the pathogenesis of some of the more disabling diseases of our time.

II. EXPERIMENTAL TECHNIQUES

The experimental techniques to be described were used not only to study the nature of the proteoglycan PP-L, but also to gain experience in as many different biochemical techniques as possible. The methods are grouped in three different sections, viz:- analytical techniques, physical measurements and experimental methods. In general, only the procedures themselves will be described but where experiments were carried out which themselves resulted in modifications of the experimental technique they will be described in the appropriate section. The majority of the results will be recorded in the Results and Concluding Discussion section.

A. Analytical Methods

i. Moisture Content

Samples of the different tissues to be analysed were cut into as finely a divided state as possible. This was found to be most easily carried out on the frozen tissue. For example, the bovine nasal cartilage was planed by hand into shavings, having been kept at -50°C overnight, prior to determination of the moisture content and to extraction of the proteoglycans. Where

it was necessary to prepare a finely divided sample of fibro cartilage it was found necessary to freeze the tissue in liquid air and crush it in a mortar and pestle. This technique was used by Barker, Guy and Cruickshank (1965) in their extensive analyses of various cartilaginous tissues.

The finely divided tissue or solution of proteoglycan was lyophilised and dried to constant weight, in vacuo, over concentrated sulphuric acid. This was the state of the material as it was used for the various experimental procedures. The residual moisture content was determined by the method of Eastoe and Courts (1963). A weighing bottle was heated overnight in an oven at 105°C and allowed to cool in a desiccator. A sample of the lyophilised material was added and the weighing bottle left overnight (16 hrs.) at 105°C, allowed to cool in the desiccator and reweighed.

ii. Ash Content

The 'sulphated ash' content of the samples were determined by the method of Eastoe and Courts (1963). A sample of oven-dried material was placed in a crucible and heated to 450°C for 3 hours in a furnace. It was allowed to cool and 2-3 drops of sulphuric acid (A.R.)

were added and the crucible heated at 800°C for 3 hours again. If any carboniferous material remained further sulphuric acid was added and the crucible reheated for 3 hours at 800°C. The ash content was expressed as a percentage of the moisture-free material.

iii. Hydrolysis

Before the connective tissue constituents can be analysed, they must be broken down into the basic units of which the proteins and polysaccharides are built up. No one method exists which can quantitatively release all the amino acids, uronic acids and hexosamines from a single sample of tissue. One can utilise optimal conditions for the release of one of the constituents or compromise, and liberate as much of the constituents as possible, correcting for any losses or other inadequacies of the method. When a tissue or compound was being hydrolysed in order to estimate one particular constituent, acid hydrolysis was used. When the amino acid, hexosamine and uronic acid contents of the same sample were being analysed, resin hydrolysis was performed. The main assays performed were for uronic acid, hexosamines, hydroxyproline and total amino acid content. The hydrolytic procedures used will be discussed under

these four subheadings and the method used for both single and collective assays will be described.

iv. Uronic Acids

Aqueous solutions of uronic acid-containing polysaccharides may be assayed directly for their uronic acid content and this was performed on 0.01% solutions of the proteoglycans. However, certain polysaccharides give, for some unknown reason, significantly low or high results when assayed directly. For example, heparin gives a 60% higher result than would be expected from its uronic acid content (Dische, 1947). Acid hydrolysis can be used to liberate the uronic acids (2 hours hydrolysis with 2N hydrochloric acid at 100°C is frequently utilised) but as they are so readily decarboxylated to pentoses by acid, resin hydrolysis was utilised in all analyses of tissues for their uronic acid content.

Resin hydrolysis was performed using Dowex 50 x 8, 200-400 mesh, in the H^+ form by, essentially, the method of Anastassiadis and Common (1958). The Dowex 50 was washed alternately with 2N sodium hydroxide and with 2N hydrochloric acid. After the last hydrochloric

acid wash it was washed with water and suspended in the minimum quantity of 0.05N hydrochloric acid to give a stiff slurry. After use, the resin was regenerated in the above manner except when it had been used for hydrolysing tissues for less than the optimum time necessary for the liberation of hydroxyproline. Thus, if the resin was used to optimally release uronic acid, fragments of insoluble collagen and elastin remained which would not be removed during the regeneration process. These proteins were removed by autoclaving the resin with excess of 4N-hydrochloric acid for 3 hours at 15 lbs./sq. in. and cycling the resin through several changes of sodium hydroxide and hydrochloric acid.

The hydrolyses were performed in 30 ml. Pyrex culture tubes. The necks of these tubes were ground dead flat with an oil stone and the plastic caps fitted with Teflon washers; these modifications are essential to avoid the occasional leakage which occurred if unmodified tubes were used. At least twenty-five times the tissue weight of resin was used. The hydrolyses were carried out in an oven at 100°C which was fitted with a device which enabled four tubes to be

mixed by revolving them end over end, at ten revolutions per minute. After cooling, the uronic acids, hexosamines and amino acids were eluted from the resin. Preliminary experiments were carried out using the method of Anastassiadis and Common (1961) which attempts to elute the uronic acid differentially from the hexosamines and hydroxyproline. The resin slurry was transferred to a column (14 mm. internal diameter) with 20 ml. of water; this eluate was collected into a conical flask and is referred to as Water Eluate I. A 50 ml. volumetric flask was placed under the column and the amino acids and hexosamines were eluted with 20 ml. of 2N-hydrochloric acid and the excess acid was removed by washing the column with 10 ml. of water, this eluate being collected into the same volumetric flask. This flask was then removed and replaced by a second 50 ml. volumetric flask. The fraction referred to as Water Eluate I was applied to the column and the effluent collected. The column was washed with a further 20 ml. of water; this volumetric flask was made up to the mark and uronic acid assays performed on this solution. The first 50 ml. volumetric flask was again placed under

the column and the re-absorbed amino acids and hexosamines eluted with 20 ml. of 2N-hydrochloric acid. This flask was made up to the mark and the contents assayed for hexosamines and amino acids; attempts to differentially elute the amino acids and hexosamines were unsuccessful (Anastassiadis and Common, 1958). In order to determine whether this differential elution was complete, both fractions were assayed for uronic acids, amino acids and hexosamines. The results, in Table II, are the results obtained using the above method on samples of bovine nasal and articular cartilage.

Table II

Nasal Cartilage	Hydroxyproline (mg./gm.wet wt.)	Hexosamine (mg/gm wet wt.)	Uronic Acid (mg./gm. wet wt.)
Water Eluate	0.0	0.077	1.23
Acid Eluate	4.6	18.4	1.02
Articular Cartilage			
Water Eluate	0.0	0.056	1.34
Acid Eluate	15.8	10.21	0.45

It is apparent that although good separation of the hydroxyproline and hexosamine occurred, considerable

quantities of uronic acid were found in the acid eluate. It was decided to elute the resin with 2N-hydrochloric acid and estimate all three substances in the single solution. The method adopted was as follows:- the resin slurry was poured into the column and the Pyrex tube washed with 30 ml. of 2N-hydrochloric acid. The column was then eluted with two 30 ml. portions of 2N-hydrochloric acid into a 100 ml. volumetric flask, to which had been added two drops of 1:1000 thiomersal (Merthiolate. Lilly), and made up to the mark with distilled water. Uronic acid was estimated directly on the solution - samples were taken to dryness three times in a rotary film evaporator at 40°C and made up to a known dilution for estimation of hexosamine and hydroxyproline.

Preliminary experiments were carried out to confirm that all of the uronic acid, hexosamine and hydroxyproline was eluted from resin by the above procedure. Samples of bovine nasal and articular cartilage and human geniculate meniscal cartilage were subjected to resin hydrolysis and the resin eluted with 90 ml. of 2N-hydrochloric acid as indicated in the previous paragraph. The resin was then re-eluted with two 50 ml. portions of

2N-hydrochloric acid. All three eluates were assayed for uronic acid, hydroxyproline and hexosamine. The results are shown in Table III. Thus this method of elution appears to elute all the detectable hexosamine and hydroxyproline and over 99% of the uronic acid.

It was next necessary to determine the optimal conditions of hydrolysis. Samples of dried bovine nasal and articular and human meniscal cartilage were hydrolysed for varying time intervals, the resin eluted with 100 ml. 2N-hydrochloric acid and each hydrolysate assayed for uronic acid, hexosamine and hydroxyproline. The results are given in Table IV.

TABLE III

Eluate Volume	Articular Cartilage	Nasal Cartilage	Meniscal Cartilage
	Uronic Acid	Uronic Acid	Uronic Acid
ml.	(mg./gm. dried tissue)	(mg./gm. dried tissue)	(mg./gm. dried tissue)
100	6.2	15.1	1.1
50	0.061	0.069	0.0
50	0.022	0.051	0.0
	Hydroxyproline	Hydroxyproline	Hydroxyproline
	(mg./gm. dried tissue)	(mg./gm. dried tissue)	(mg./gm. dried tissue)
100	15.8	9.05	25.0
50	0.0	0.0	0.0
50	0.0	0.0	0.0
	Hexosamine	Hexosamine	Hexosamine
	(mg./gm. dried tissue)	(mg./gm. dried tissue)	(mg./gm. dried tissue)
100	6.82	26.7	7.2
50	0.0	0.0	0.0
50	0.0	0.0	0.0

TABLE IV

	Uronic acid (mg./gm. dry tissue)	Hydroxyproline (mg./gm. dry tissue)	Hexosamine (mg./gm. dry tissue)
Articular Cartilage			
Time of hydrolysis (hrs.)			
1	40.2	-	-
2	44.8	-	-
3	42.8	-	-
6	40.9	-	-
9	35.1	-	29.1
18	30.2	50.0	35.0
24	24.1	75.1	40.2
30	21.4	87.0	42.4
36	18.2	86.8	42.0
Nasal Cartilage			
Time of hydrolysis (hrs.)			
1	110.0	-	-
2	133.0	-	-
3	129.0	-	-
6	120.0	-	-
9	114.0	5.1	51.1
18	68.5	10.0	100.0
24	53.6	15.1	120.0
30	46.7	15.8	128.0
36	37.2	15.9	127.0

TABLE IV (Contd.)

	Uronic acid (mg./gm. dry tissue)	Hydroxyproline (mg./gm. dry tissue)	Hexosamine (mg./gm. dry tissue)
Meniscal Cartilage			
Time of hydrolysis (hrs.)			
1	4.98	-	-
2	6.7	-	-
3	6.0	-	-
6	5.9	-	-
12	5,4	80.5	8.1
18	5.0	91.5	10.5
24	4.5	97.0	11.4
30	3.96	98.0	11.4
36	3.5	98.1	11.0
42	3.2	97.5	10.0

It was concluded that optimal release of uronic acid occurred after two hours hydrolysis and of hydroxyproline and hexosamine after thirty hours hydrolysis. These results are in reasonable agreement with the studies of Haab and Anastassiadis (1961) who showed that thirty-six hours hydrolysis led to optimal hydrolysis of oviduct magnum. They reported, however, only a 20% loss in uronic acids after thirty hours hydrolysis as against a 40 - 50% loss

noted for various cartilaginous tissues in this work. Using the differential elution technique, any oligsaccharides from the partial hydrolysis of the polysaccharides will be eluted with the amino acids and hexosamines giving low readings for the uronic acid content. This could account for the findings of Thirkell and Henderson (1963) that optimal release of uronic acid from calf skin occurred after 30 - 36 hours and that the uronic acid content of calf skin was lower than that previously reported. Their values for hexosamine and hydroxyproline content were in good agreement with those quoted in the literature; optimal hydrolysis was found to occur after 30 - 36 hours. These times were utilised in the hydrolysis of the various cartilaginous tissues to be reported in the results section.

v. Hexosamines

Hydrolysis of samples of tissue for the estimation of hexosamines were carried out by the method of resin hydrolysis described above. Acid hydrolysis was performed on samples of proteoglycan being subjected to hexosamine analysis. The hydrolyses were performed in 30 ml. Pyrex

tubes with at least a two-hundred-fold excess of 4N-hydrochloric acid A.R. The tubes were flushed out with nitrogen before closing them and they were heated for 8 hours at 105°C. After cooling the hydrolysates were taken to dryness three times, to remove the hydrochloric acid, in a rotary film evaporator at 40°C and made up to known dilution with distilled water.

vi. Hydroxyproline

When samples of materials were to be assayed solely for hydroxyproline, strong acid hydrolysis was used. Samples of material were placed in the Pyrex culture tubes, a two-hundred-fold excess of constant boiling point (5.7N) hydrochloric acid, which had been doubly distilled over stannous chloride, was added, the tube flushed out with nitrogen and sealed. Hydrolysis was carried out for 20 hours at 110°C. A recent study of the liberation of hydroxyproline from gelatine by acid hydrolysis indicates that only 12 hours hydrolysis is necessary at 100°C and that optimum yield occurs after 1 hour hydrolysis at 130 - 150°C, using a similar hydrolytic technique to that described above (Pashley, Claycomb and Summers, 1966). It was also shown that significant destruction of the hydroxyproline only occurred

after prolonged hydrolysis (48 hours at 150°C). Estimation of hydroxyproline, in samples of tissues that were also to be assayed for uronic acid and hexosamines, were performed on resin hydrolysates.

vii. Amino Acid Content

Hydrolysis of proteins and protein complexes on which quantitative estimations of all amino acids are required necessitates special consideration. Acid hydrolysis for 24 hours with 6N-hydrochloric acid at 110°C is usually performed and gives reliable results for most of the amino acids; some loss of the amino acids, particularly serine and threonine, occurs but this can be corrected for. A very considerable loss of tryptophan occurs and it is necessary to hydrolyse a sample of tissue with alkali before the estimation of this amino acid. It is hoped that resin hydrolysis would solve the problem of loss of certain amino acids during the hydrolysis, especially when there are considerable quantities of carbohydrate and/or lipid present. Paulson, Deatherage, and Almy (1953) showed that 48 hours hydrolysis with Dowex 50 at 100°C almost completely degraded the proteins. Improved yields of aspartic acid, serine and threonine were noted but the

resin was found to have serious disadvantage. It appeared to preferentially cleave certain peptide bonds particularly those adjacent to the above three amino acids. Peptide bonds adjacent to cystine and valine were found to be particularly resistant to resin hydrolysis. Recovery of glutamic acid was found to be particularly low and it was shown that twenty-five per cent of this amino acid was converted into pyrrolidone-carboxylic acid. Pöhm (1961) has modified this technique and claims to have quantitatively liberated tryptophan, phenylalanine and lysergic acid from crude ergot alkaloids by resin hydrolysis with IRC 112(H^+) in the presence of 80% ethanol at 90-95°C for 6 to 10 hours. The value of this technique in the hydrolysis of proteins and protein complexes, if confirmed, would be significant. The use of water-soluble polystyrene sulphonic acid (Painter and Morgan, 1961) in hydrolysis prior to amino acid analysis also requires further investigation.

Acid hydrolysis was carried out with a two-hundred-fold excess of constant boiling point hydrochloric acid at 110°C in sealed Pyrex tubes. Samples were hydrolysed for 24, 36 and 72 hours and subjected to amino acid

analysis. Optimal values were used in the calculation of the amino acid composition, or when necessary a correction factor was calculated assuming destruction of the amino acids followed first order kinetics. Alkaline hydrolysis was carried out to obtain reliable results for the tryptophan content of the proteoglycan. The method used was that of Brenner, Niederwieser and Pataki (1965) and is as follows:-

10-20 mg. of material, 130 mg. of hydrated barium hydroxide and 2 ml. of water were mixed in a small Pyrex tube. This was flushed out with nitrogen, sealed and heated for 24 hours at 125-130°C. The reaction mixture was adjusted to pH 6 with 2N-sulphuric acid, heated in a boiling water bath for 10 minutes and centrifuged to remove the barium sulphate. The residue was washed with 5 ml. of water, the supernatant and washings combined and taken to dryness, *in vacuo*, and assayed in the amino acid analyser.

viii. Preservation of Hydrolysates.

Where possible the hydrolysates were stored in a dried state in a refrigerator. Aqueous solutions of hydrolysates rapidly become contaminated with bacteria and the use of thiomersal as a preservative was investigated

Its bactericidal activity was demonstrated in the following experiment. Two batches of nutrient agar (Oxoid) were sterilised by autoclaving; to one, hundred ml. sample had been added two drops of 1 in 1000 thiomersal (Methiolate. Lilly), the other sample acted as a control. Sterile petri dishes were filled with samples of the agar using normal bacteriological techniques. Petri dishes, filled with thiomersal-treated agar and control dishes were exposed on the laboratory bench for varying periods, incubated at 37°C for 24 hours and the number of colonies counted. The results are shown in Table V.

TABLE V

Time exposed (hours)	Control (colonies/plate)		Thiomersal -treated (colonies/plate)	
3	16,	10	0,	0
6	30,	25	0,	0
9	45,	80	1,	0

After storing at room temperature for four weeks, it was found that the thiomersal-treated agar had lost its bacteriostatic effect. This concentration of thiomersal was shown not to interfere with any of the

assay procedures used and two drops thiomersal per 100 ml. of hydrolysate were routinely added.

ix. Assay of Uronic Acid

Most of the uronic acid assay procedures are modifications of the original Dische (1947) procedure in which the uronic acid is heated with 87% sulphuric acid to yield 5-carboxy-2-formylfuran which gives a coloured product with alcoholic carbazole (Stutz and Duvel, 1956). The modification used was that of Bowness (1957) and is as follows:-

Reagents

a) Sulphuric acid 1.84 sp. gr. M.A.R. grade - ordinary grade and A.R. grade sulphuric acid were found to be unsatisfactory as they gave a brown discoloration during the heating stage.

b) Carbazole 0.2% in 99% ethanol, stored at 4°C for one month - after this time the solution had a blue tinge. Several different samples of carbazole were tried but all gave some 'browning' when mixed with the 87% sulphuric acid, even after three crystallizations from benzene.

c) Glucuronolactone standard - a stock standard solution of 1 mg./ml. was kept frozen at -20°C - working standards

of 0.1 mg./ml. were prepared weekly and stored at 4°C.

Method

1 ml. aliquots of the unknown solutions and of a series of standard solutions from 10 - 100 µg./ml. were placed in Pyrex test tubes in an ice bath. 6 ml. of ice-cold sulphuric acid were rapidly added and the tubes shaken. The tubes were heated for 10 minutes in a water bath at 100°C, cooled in an ice bath, and then allowed to warm to room temperature. 0.2 ml. of the carbazole solution was added to the tubes: 0.2 ml. of 99% ethanol was added to the appropriate 'blank' tubes. After mixing, the tubes were placed in an incubator at 26°C for two and a half hours and the absorption at 525 mµ. read in 1 cm. glass cells in a Unicam SP600 spectrophotometer. All assays were performed in triplicate and a series of standards was estimated on every occasion. The unknown solutions were read in the spectrophotometer against 1 ml. aliquots of the unknown solution, treated as above, but to which 0.2 ml. of 99% ethanol had been added, instead of the carbazole solution. The standards were read against appropriate water blanks. Water blanks were also used to which carbazole was added. By this means it was hoped to correct for the brown coloration produced

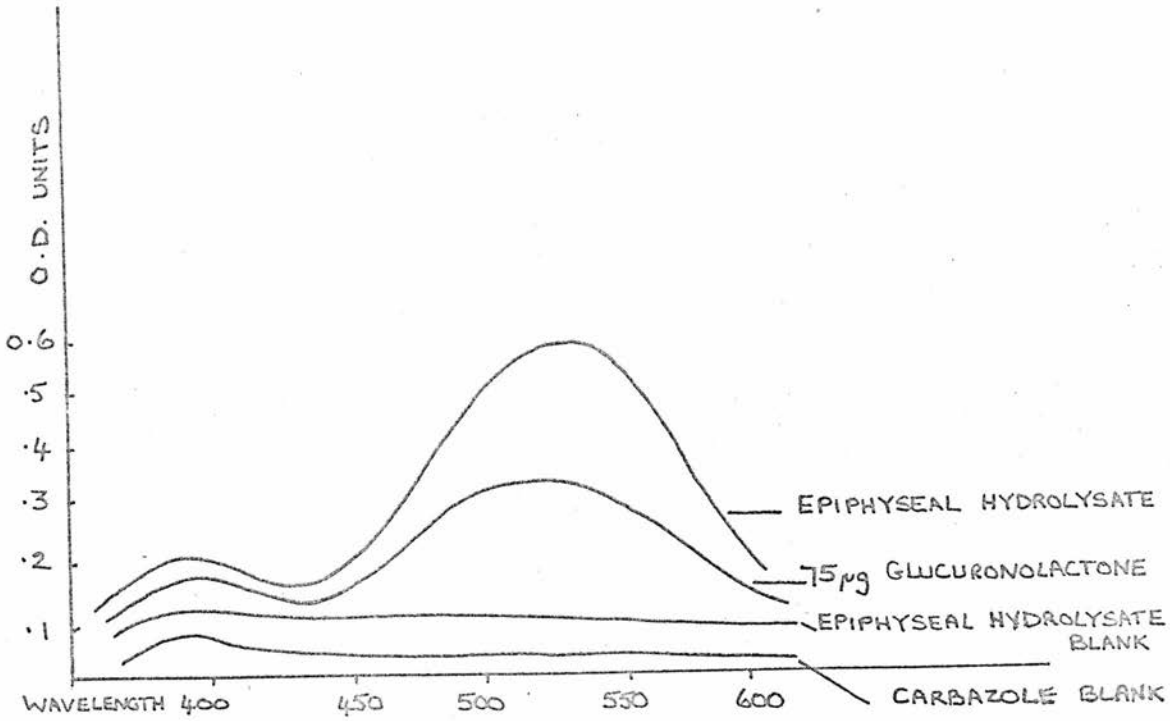


FIG VI ABSORPTION SPECTRA OF CHROMOGENS BETWEEN GLUCURONOLACTONE AND EPIPHYSEAL HYDROLYSATES AND CARBAZOLE. SPECTRA OF HYDROLYSATE AND CARBAZOLE BLANK ALL READ AGAINST 87% SULPHURIC ACID

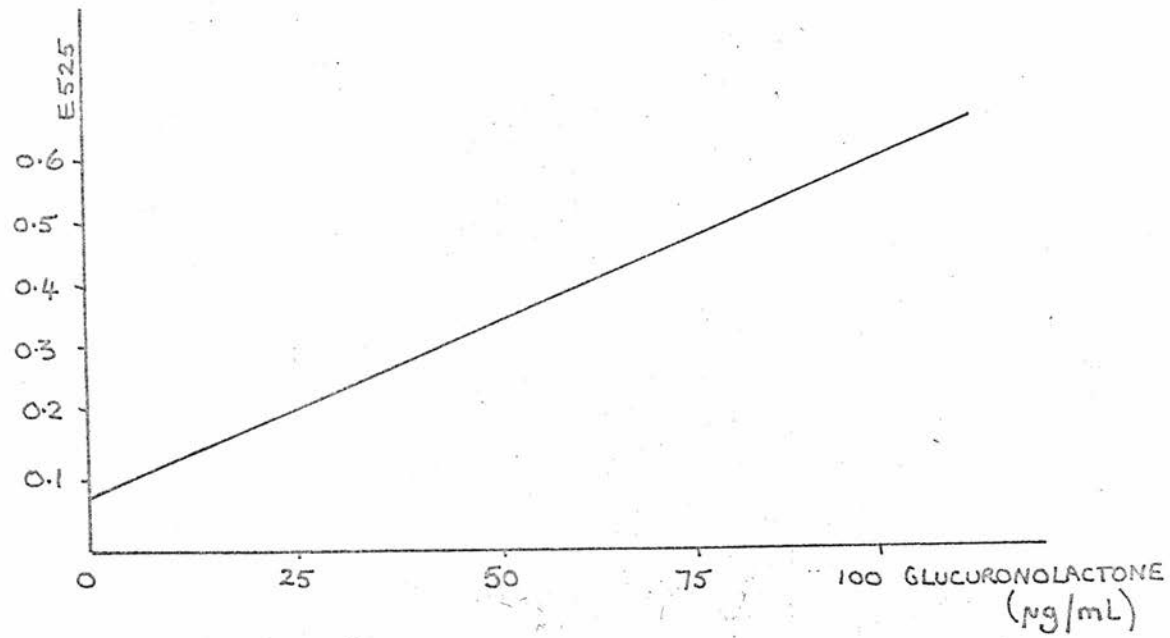


FIG VII TYPICAL STANDARD CURVE OBTAINED USING BOWNESS METHOD OF UROMIC ACID ASSAY

between carbazole and sulphuric acid and the coloration produced when the hydrolysate was heated with the sulphuric acid. Absorption spectra for both a standard solution of glucuronolactone, for a cartilage hydrolysate and of the hydrolysate and carbazole blanks are shown in the adjoining figure (Fig. VI). Although the latter two solutions do give a significant absorption at 525 mμ., they do not give an absorption maximum in this region and the use of dichromatic readings can be employed to correct for these side reactions.

The method is reasonably linear over the range 10 - 100 μg./ml. (Fig. VII). It is important to avoid any contamination due to lint etc., as this can give charring and thus abnormally high readings. Attempts to distil the chromogen, 5-carboxy-2-formylfurfuran into a solution of carbazole and thus eliminate the interference by side reactions were completely unsuccessful. In retrospect, the modification of Bitter and Muir (1962) was perhaps preferable to the method of Bowness (1957). The advantages claimed for this procedure, which includes 0.025M sodium tetraborate in the concentrated sulphuric acid, are a two-fold increase of sensitivity, more rapid and more stable colour development, greater reproducibility

and reduced interference by inorganic ions. However, the method of Bowness (1957) was developed for the estimation of uronic acids in urine and this assay procedure has twice the effective range of the Bitter and Muir procedure.

x. Hexosamine (amino-glycan)

The ideal method for the estimation of hexosamines should be sensitive and accurate, give identical extinction coefficients with the common amino-glycans and should suffer a minimum of interference from amino acids and proteins. It is on these last two factors that most procedures err. After preliminary experiments, using the Elson-Morgan and the indole-hydrochloric acid methods (Dische, 1955), the definitive method adopted was that of Cessi and Pilego (1960). This method is a modification of the Elson-Morgan method and makes use of the observation of Schloss (1957) that 2-methyl-pyrrole*, the main chromogen produced during the interaction of the amino-glycan and acetyl-acetone, can be steam distilled. The volatile chromogens are distilled into Erlich's solution forming a stable

---

* There is, however, dispute as to the nature of the main volatile chromogen (Gonzalez, Sanchez and de Rey, 1965).

coloured product with an absorption maximum at 548 mμ. The method is highly specific, being unaffected by any amino acids except by hydroxyproline to a very small degree; this may account for the 'low' value for the hexosamine content of ovomucoid obtained by the Cessi procedure as compared with that obtained using the original Elson-Morgan procedure (Johansen, Marshall and Neuberger, 1960).

The previous modifications of the Elson-Morgan technique give over-estimates, as the amino acids, particularly lysine, give red products in the presence of neutral sugars (Neuberger and Marshall, 1966). It was found to be very suitable for the dark hydrolysis products produced by the various cartilages and proteoglycans studied in this work. The molar extinction coefficients and absorption maxima for galactosamine and glucosamine are identical (Cessi and Pilego, 1960) which makes it a very suitable method for studying tissues with different keratan sulphate/chondroitin sulphate ratios. The colour is independent of the presence of salt up to 5M sodium chloride (Neuberger and Marshall, 1966) but accurate control of the pH of the acetyl solution is important and as a check that the acid in the hydrolysates had been fully removed one

drop of a 0.1% solution of phenolphthalein solution in 99% ethanol was added to each tube; this was shown not to interfere with the procedure in any way. The method adopted is as follows:-

Reagents

a) Acetyl-acetone (B.D.H.) - this reagent was redistilled to give a colourless solution (B.P.138 - 140°C) and 1 ml. dissolved in 100 ml. of 0.5N sodium carbonate - sodium bicarbonate buffer containing 0.1M sodium chloride, immediately before use. The pH of this solution should be 9.8.

b) Ehrlich's reagent - 80 mg. of p-dimethylaminobenzaldehyde (A.R.) and 3.5 ml. concentrated hydrochloric acid were made up to 100 ml. with absolute ethanol. This reagent is stable for several weeks if stored at 4°C.

Method

2 - 50µg. of hexosamine in 2 ml. of water were placed in a 30 ml. Pyrex culture tube. 1 drop of 0.1% ethanolic phenolphthalein was added together with 5.5 ml. of freshly prepared acetyl-acetone reagent, whence the contents should be bright pink. 4N-sodium-hydroxide was added if the contents were too acidic. The caps were replaced, the tubes mixed and then heated in a

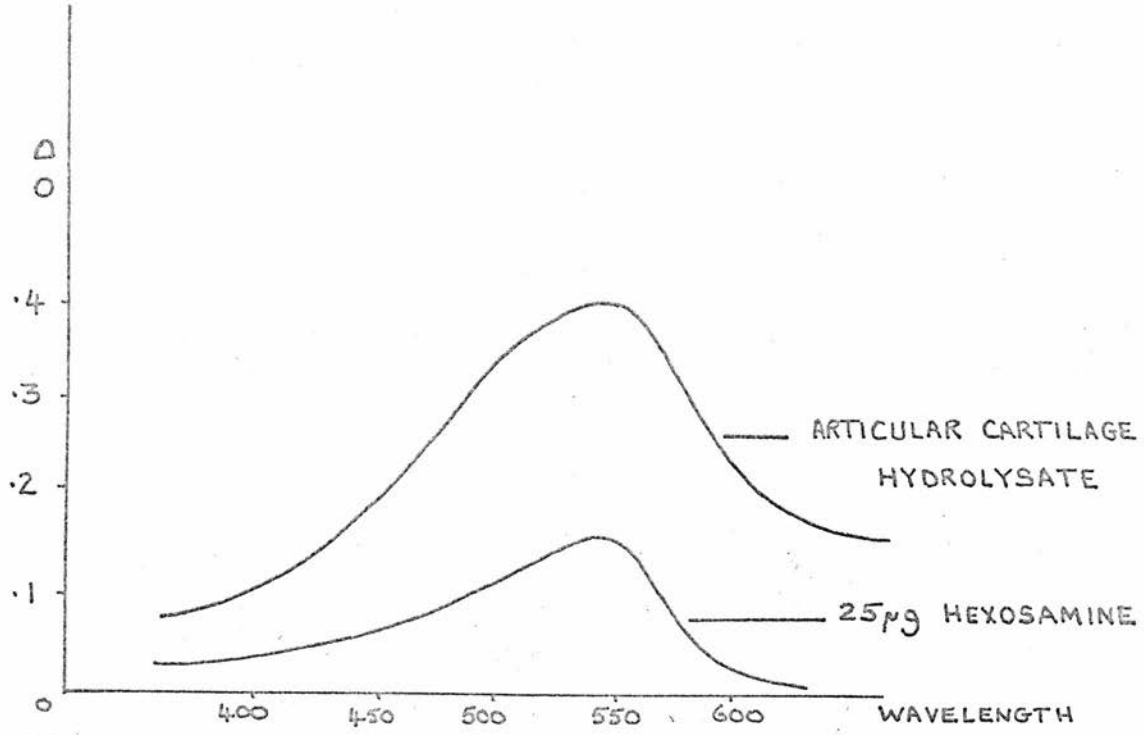


FIG VIII ABSORPTION SPECTRA OF CHROMOGEN PRODUCED IN THE CESSI PROCEDURE — READ AGAINST REAGENT BLANK.

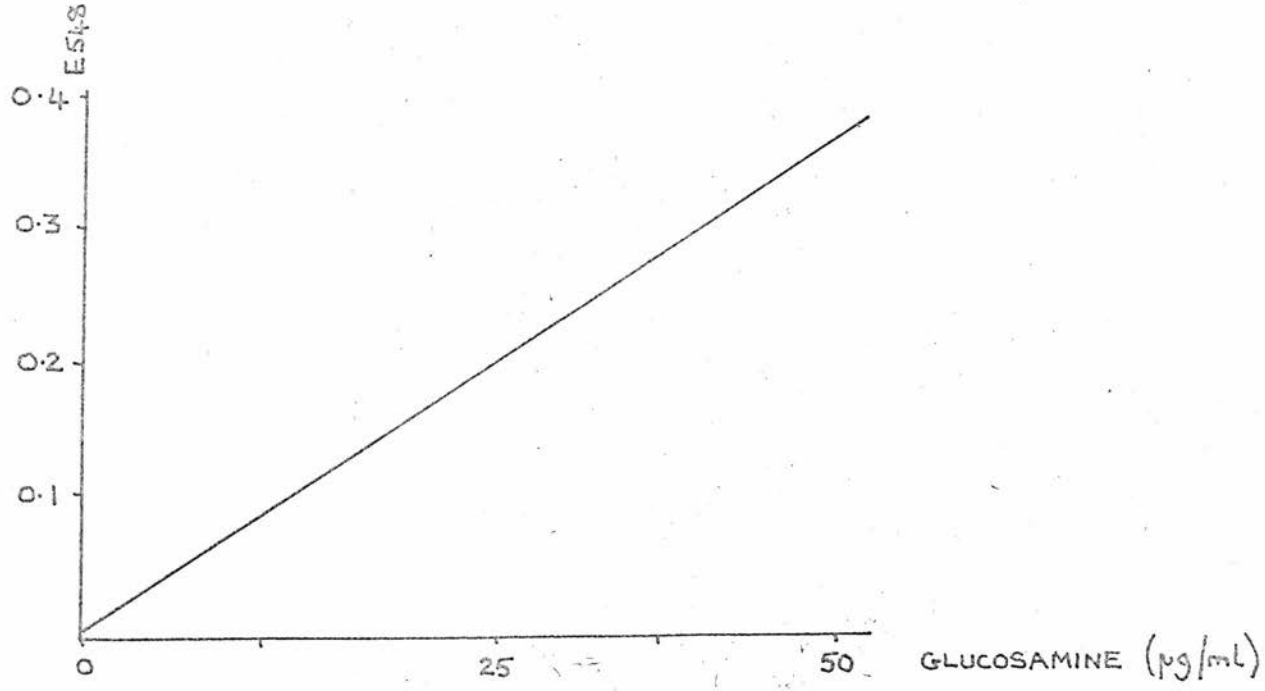


FIG IX TYPICAL STANDARD CURVE OBTAINED BY CESSI PROCEDURE FOR HEXOSAMINE ASSAY

boiling water bath for twenty minutes. The tubes were then immersed in an ice bath before distillation. The chromogens are stable for approximately 30 minutes in this state (Schloss, 1951). The contents of the tubes were washed into a 100 ml. round-bottom flask with three 2 ml. aliquots of water - dispensed with a Cornwall automatic pipette. A piece of porous pot was added and the chromogen distilled into 8 ml. of Ehrlich's reagent using the small distillation apparatus recommended, at a rate of 1 ml. per minute. The distillation was continued until the Ehrlich's solution had been made up to 10 ml. in a measuring cylinder. The optical density was read at 548 mμ. in 1 cm. glass cells in a Unicam SP600, after standing at room temperature overnight. A series of standards and a reagent blank were estimated in every assay performed. The absorption spectrum of the final coloured product(s) and a typical calibration curve are shown in figures VIII and IX respectively.

Ogston, in a previous paper (Ogston and Stanier, 1950) showed that the estimated amount of hexosamine in their hyaluronic acid complex was less than the theoretical amount and in a recent paper (Ogston, 1964) this observation has been further investigated. He

showed that when glucosamine was hydrolysed in the presence of protein, with 4N-hydrochloric acid at 105°C, there was an apparent 25% loss of glucosamine whilst there was only a 10% loss if the glucosamine was similarly treated alone. This apparent loss of glucosamine appeared to be independent of the amount of glucosamine present and occurred during the reaction with the alkaline acetylacetone. He claimed that this loss was a consequence of the presence, in the alkaline acetylacetone reaction stage, of products of the acid hydrolysis. This loss appeared to occur independently of the method of hydrolysis or of the mode of reaction of the chromogen with the Ehrlich's reagent. It would thus appear that some partial hydrolysis product of the protein interferes with the reaction between glucosamine and alkaline acetylacetone. It is suggested that a side reaction occurs between these partial hydrolysis products and the acetylacetone, deviating this latter compound from its reaction with the hexosamine. The presence of excess acetylacetone would therefore be expected to reduce this apparent loss of glucosamine and it would be expected that the loss would be greater if larger rather than smaller amounts of protein

hydrolysis products were present. Ogston did not investigate these points but it is interesting to note that the amount of acetylacetone he used was less than that advocated in all other methods (Fig. X), which estimate hexosamines over a similar range.

Fig. X

Method	Amount of acetyl-acetone added per assay. (ml.)	% acetyl-acetone in reagent.
Ogston (1964)	0.01	2.0
Rondle and Morgan (1954)	0.02	2.0
Blix (1948)	0.03	1.5
Schloss (1951)	0.049	0.98
Boas (1953)	0.05	2.0
Cessi and Pilego (1960)	0.055	1.0
Svennerholm (1956)	0.4	4.0

Immers and Vasseur (1950, 1952) have studied various parameters in modifications of the Elson-Morgan reaction and have shown that if the concentration of acetylacetone reaction is increased from 0 - 5%, the colour formed by glucosamine increases from zero to a limiting value.

They have also shown that chromogens are formed if a mixture of lysine or glycine and glucose is treated with alkali. Cessi and Pilego (1960) have shown that the lysine - glucose alkali chromogen is non-volatile, but they have not excluded the possibility of any other volatile non-hexosamine chromogens being formed.

It was decided to investigate and if possible verify some of the points raised by Ogston. Samples of articular cartilage and nasal cartilage were hydrolysed to release their hexosamines and hexosamine assays were performed using the Cessi procedure. To samples of the hydrolysates, glucosamine was added and the solutions re-assayed for their hexosamine content. The differences in optical density due to the added glucosamine was calculated and compared with that obtained if a sample of the glucosamine alone was assayed. Fig. XI demonstrates that the optical density of the 25 µg. of glucosamine assayed alone or in the presence of the cartilage hydrolysates is identical.

Fig. XI

Solution assayed	E548	Difference in O.D. due to 25µg.glucosamine
i. 25µg. glucosamine	0.141	0.141
ii. Articular cartilage hydrolysate	0.031	-
iii. A.C. hydrolysate + 25µg. glucosamine	0.173	0.142
iv. Nasal cartilage hydrolysate	0.074	-
v. N.C. hydrolysate + 25µg. glucosamine	0.213	0.139

These results are in complete disagreement with those of Ogston who found a significant decrease in the difference in optical density due to the added glucosamine if assayed in the presence of hyaluronic acid, glucuronic acid and bovine serum hydrolysis products.

The amount of interfering material present in the dilutions of the cartilage hydrolysates used in the assay will be very small and it was decided to investigate the effect of larger quantities of protein breakdown products on the yield of chromogen by the Cessi procedure. Samples of bovine haemoglobin (Sigma), ossein gelatine (British Glues) and blood albumen (B.D.H.) were hydrolysed in 4N-hydrochloric

acid under the conditions required to liberate hexosamines. The hydrolysates were taken to dryness to remove the hydrochloric acid and made up to a known volume, such that 1 ml. of the solution contained the hydrolytic products of 50 mg. of the original protein. 25 µg. of glucosamine were assayed in the presence of varying amounts of the protein hydrolysates using the Cessi procedure. Fig. XII shows the effect of the protein hydrolysate on the apparent yield of hexosamine, after correcting for the small amount of hexosamine present in the original protein. The results are the means of two independent experiments performed in duplicate. It would appear that increasing quantities of the gelatine hydrolysate and, to a lesser extent, the haemoglobin hydrolysate, reduces the yield of chromogen from the glucosamine. On the other hand, the albumen hydrolysate appeared to produce two opposing effects. In small quantities, the apparent yield of glucosamine was increased, but with larger quantities of the hydrolysate this apparent enhancement tended to fall off. The cause of these phenomena are not clear. It is suggested that the decreasing recovery of glucosamine with increasing protein concentration is due to deviation of the acetyl-acetone from its intended reaction with the hexosamine. However, when the above experiment with the albumen hydrolysat

Fig. XII

Protein	Weight of protein Hydrolysate (mg.)	Recovery of added 25µg. of glucosamine (µg.)
Haemoglobin	0	25.0
	2	24.6
	5	24.4
	10	24.0
	20	24.0
Gelatine	0	25.0
	5	24.5
	20	20.1
	30	16.6
	40	9.8
Albumen	0	25.0
	5	40.1
	10	51.9
	20	59.6
	30	59.0
	40	44.8
	50	30.8

was performed using a 2% alkaline acetylacetone solution, rather than the usual 1% solution, similar results were obtained. No adequate explanation is therefore offered for these results but since the amount of the protein hydrolysis products present were in a thousand-fold excess over the amount of hexosamine, the significance of the findings in the analysis of connective tissues is unclear. Since the experiments using samples of cartilage did not confirm Ogston's findings and as most of the hydrolyses were performed using a resin where humin formation and other side reactions might be expected to be minimal, this aspect of the work was not pursued further. It is, however, probably worthy of further study and does indicate that certain estimations of the amount of hexosamines in certain glycoproteins may need to be interpreted with caution.

xi. Estimation of hydroxyproline

The method adopted was that of Cessi and Serafini-Cessi (1964). This method is an improvement on the original Neuman and Logan (1950) procedure in that it is not affected by tyrosine and tryptophan even if present in a thousand-fold excess, whereas in the Neuman and Logan procedure these amino acids give a 2% interference. As the chromogen is steam distilled from the alkaline oxidising solution into the Ehrlich's solution, interference due to humin formation, turbid solutions, etc., is avoided. Similarly, the use of internal standards as utilised in the Bergman and Loxley (1961) procedure and the double extraction technique as advocated in the Prockop and Udenfriend (1960) modifications are both rendered obsolete by the Cessi method.

Reagents

a) A stock standard of hydroxyproline 1 mg./ml. in 0.01N-hydrochloric acid is stored at -15°C. This is diluted daily to give a working standard of 10 µg./ml.

b) 2.5N Sodium hydroxide.

c) 0.05M Copper sulphate.

d) 30% Hydrogen peroxide A.R. stored at 4°C and diluted to give a 6% solution immediately before use.

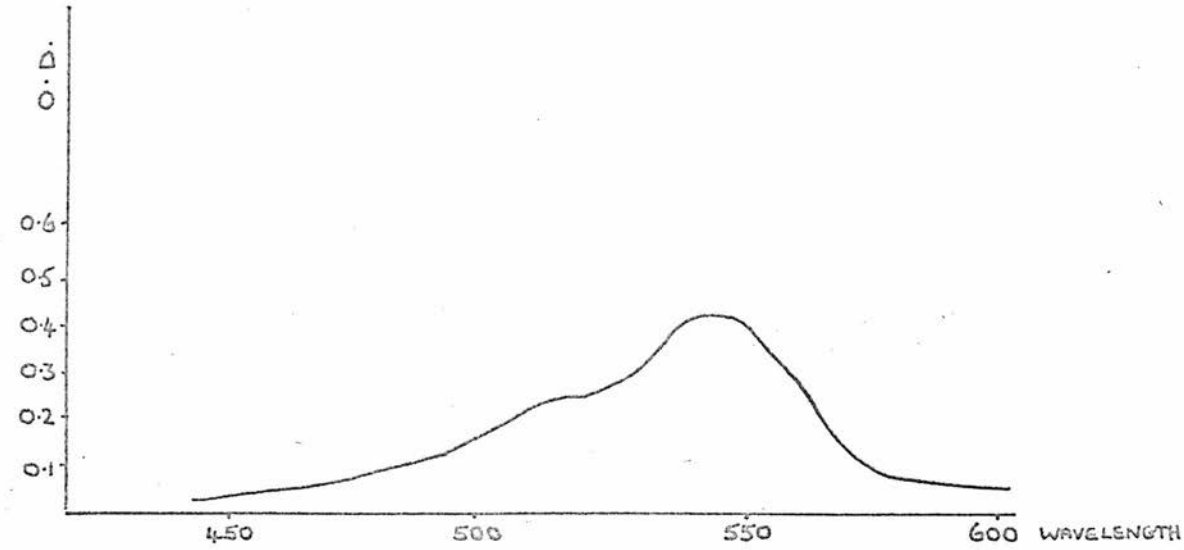


Fig XIV ABSORPTION SPECTRA OF CHROMOGEN IN THE CESSI AND SERAFINI-CESSI PROCEDURE FROM 10 µg HYDROXY-PROLINE — READ AGAINST REAGENT BLANK

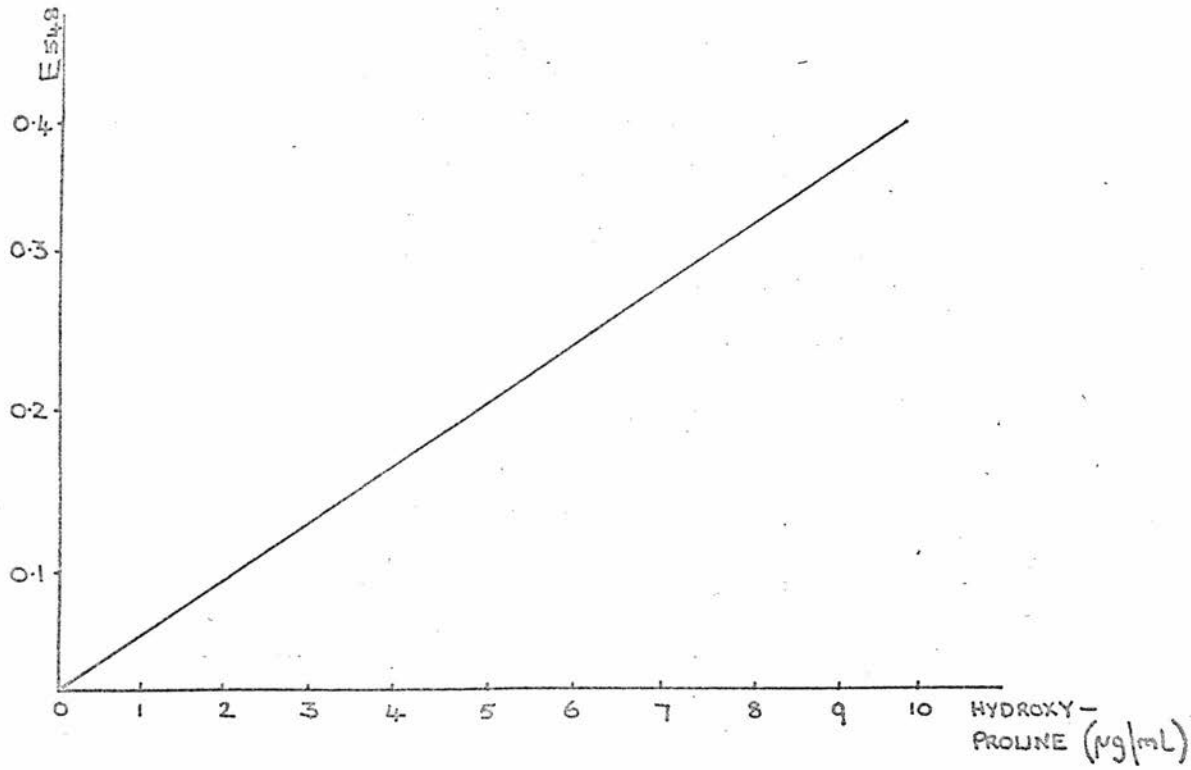


Fig XIII TYPICAL STANDARD CURVE OBTAINED IN HYDROXY-PROLINE ASSAY

e) 3N Hydrochloric acid.

f) Saturated aqueous potassium chloride.

g) Stannous chloride crystals.

h) Ehrlich's reagent - prepared daily. 1 gm. of p-dimethylaminobenzaldehyde (A.R. grade) is dissolved in 55 ml. of glacial acetic acid (A.R. grade) and 10 ml. of 10N sulphuric acid (A.R. grade). The use of A.R. grade reagents in making up the Ehrlich's reagent is important if high blank readings and cloudy solutions are to be avoided.

Method

To 1 ml. of hydrolysate and to a series of standards from 1 to 10 µg. of hydroxyproline in test tubes, 1 ml. 0.05M copper sulphate and 1 ml. 2.5M sodium hydroxide were added. The contents were mixed and placed in a water bath at 40°C and after the temperature was reached, 1 ml. 6% hydrogen peroxide was added. The tubes were heated in the bath for 10 minutes with occasional shaking. They were kept in an ice bath until distilled. Immediately prior to distillation, 2 ml. saturated potassium chloride, 1 ml. of 3N-hydrochloric acid and a crystal of stannous chloride were added. The contents of the tubes were transferred to a similar micro-distillation apparatus as used in the Cessi method for

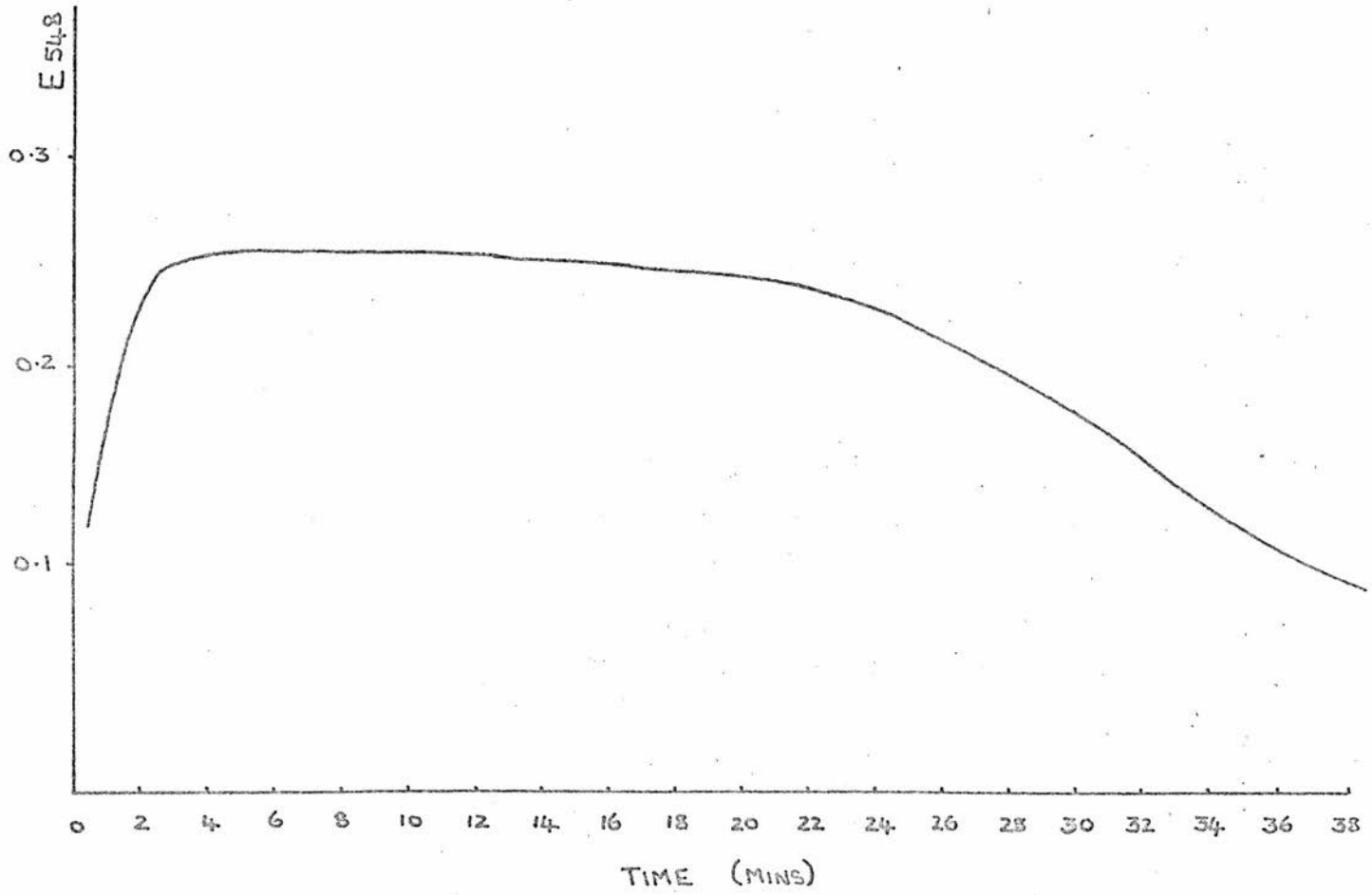


Fig XV GRAPH OF CHANGE IN E548 OF THE CHROMOGEN PRODUCED BY 5γg OF HYDROXY-PROLINE, WITH TIME.

hexosamine determination, together with three 2 ml. washings. The chromogens were distilled at the rate of 1 ml. per minute into a measuring cylinder which contained 6.5 ml. of freshly prepared Ehrlich's reagent, until the total volume was 10 ml. The contents of the cylinder were mixed and read against a reagent blank in a 1 cm. glass cell at 548mμ. in a Unicam SP600 spectrophotometer. The colour develops almost immediately and fades significantly after 20 minutes. Fig. XIII is a typical standard curve obtained. Fig. XIV is the absorption spectrum of the final product. Fig. XV is a graph of E_{548} against time indicating the almost immediate development of the colour and the fading of the colour after fifteen to twenty minutes.

xii. Amino acid analysis

These were performed on the hydrolysates using a Technicon Autoanalyser. Corrections for amino acid destruction during hydrolysis were made by plotting the content of the amino acids after hydrolysing for 24, 36 and 72 hours, and extrapolating to zero time. Tryptophane was estimated on the hydrolysate prepared by alkaline hydrolysis.

xiii. Nitrogen

The microkjeldahl assay was used. Two ml. of nitrogen-free sulphuric acid and a small quantity of catalyst were added to the sample in a Kjeldahl flask. The catalyst used was compounded of potassium sulphate; 80 parts, hydrated copper sulphate; 20 parts, and sodium selenate; 1 part. After digestion overnight (16 hours) the ammonia, liberated with 70% sodium hydroxide, was collected by steam distillation and titrated against N/70 hydrochloric acid using Tashiro's indicator. Zero errors were determined by plotting the hydrochloric acid titre against the quantity of solution taken using a series of samples for each protein.

xiv. Protein

The estimation of the protein content of glycoproteins and proteoglycans poses several difficulties (Eastoe, 1966). The Folin-Lowry method is unsatisfactory as the intensity of the colour varies from protein to protein over a range of 300%. Calibration is not feasible using an impure proteoglycan such as PP-L, particularly when one is following the purification of

a substance whose protein content decreases with purification and in which the concentration of the amino acids which contribute most to the colour reaction is variable. Measurement of the optical density at 280mμ. is similarly unsuitable.

Assay of the protein content using the biuret method lacks sensitivity and apparently gives discrepancies with glycoproteins (Eastoe, 1966). The method adopted was to estimate the total nitrogen, using the microkjeldahl procedure, of the sample and subtract the hexosamine nitrogen as estimated by the Cessi method. The nitrogen content of the protein was calculated from the amino acid analysis of the proteoglycan. To avoid false high values for the protein content it is important to correct for losses of hexosamine occurring during the hydrolysis and during the removal of the acid in the rotary film evaporator, by subjecting a sample of galactosamine hydrochloride to the analytical procedure (Neuberger and Marshall, 1966). Eastoe (1966) states that the most reliable assessment of the total protein content of a glycoprotein is the sum of the individual values for all the amino acid residues and this was performed.

xv. Collagen and Elastin content

In the analysis of the various cartilaginous tissues for their collagen and elastin content, a modification of the Neuman and Logan (1950a) procedure was used. Samples of lyophilised tissue were placed in centrifuge tubes and distilled water added (5 ml. per 100 mg. of tissue). The tubes were fitted with cotton wool plugs and foil caps and autoclaved for 3 hours at 15 lbs. per square inch. After cooling, the tubes were centrifuged and the supernatants collected. This was repeated twice. The residues were heated for 45 minutes with 10 ml. 0.1N NaOH at 100°C, cooled, centrifuged and the supernatant discarded. The combined supernatants and residues were hydrolysed with constant boiling point hydrochloric acid for 24 hours at 110°C and assayed for their hydroxyproline content using the method of Serafini-Cessi and Cessi (1964). Hydroxyproline was converted to its equivalent of collagen, using the factor 7.46. The elastin content was determined by multiplying the 'residual' hydroxyproline by the factor 52.3 (Neuman and Logan, 1950a).

B. Physical Methods

Viscosity

Accurate measurements of viscosity and estimation of the changes in viscosity with time, during chemical and enzymatic reactions, were performed using the technique of Schachman (1957). A capillary viscometer was used in a 40 litre glass tank whose temperature was very accurately controlled using a thermo-relay in conjunction with a Beckman thermometer. The circulating pump was arranged so that a stream of liquid was directed from the heating element onto the thermo-regulator. Each viscometer was fitted in a Perspex frame so that it was mounted rigidly in the tank and in an exactly reproducible position. Before use, the viscometer was cleaned by suction with distilled water, ethanol and ether, and similarly after use. Before viscometry measurements, all solutions were centrifuged at low speed to remove any dust particles. 10 ml. aliquots of the various solutions were used in every case. Readings were taken of the time taken for the meniscus to pass between the two slits on the viscometer. This was made more reproducible and less

subjective by placing a low wattage bulb to shine light down the viscometer so that as the meniscus passed the slit there was a flash of light. Readings were taken with a stop watch until successive readings agreed to within 50 m.secs. In some of the preliminary experiments and some of the enzyme experiments, two similar viscometers were set up, one containing the enzyme and substrate, the other acting as a control containing substrate alone. The results of the viscometer measurements were used in calculating the S^{o}_{20} with greater confidence than by sedimentation measurements alone, and this will be discussed under the section on ultracentrifugation. In comparing the effect of certain enzymes and reagents on the proteoglycan, the change in the relative viscosity (η / η_o) was calculated. The intrinsic viscosity, defined as lim. $c \rightarrow o$ $(\frac{\eta - \eta_o}{\eta_o} \cdot \frac{1}{c})$ was estimated for various preparations of the proteoglycans by measuring the flow times for various dilutions of the proteoglycan in 0.15M potassium chloride and plotting $(\frac{\eta - \eta_o}{\eta_o} \cdot \frac{1}{c})$ against c, where c is the concentration in gm./100 ml. η is the flow time, in seconds, for the proteoglycan solution and η_o is the flow time of the solvent, i.e. 0.15M

potassium chloride. The significance, however, of these results when applied to macromolecules is uncertain (Gibbons, 1966). The units of intrinsic viscosity are decilitres grm^{-1}.

Density

Sedimentation coefficients are generally expressed as $S^{0}_{20,w}$ i.e. the value that the material would be expected to have at infinite dilution in a solvent with the same density and viscosity as water. Since all experiments were performed at 20°C, it was only necessary to correct for any difference in the density and viscosity of the solvent (0.15M potassium chloride) from that of water using the formulae:

$$S^{0}_{20,w} = S^{0}_{20,0.15MKCl} \cdot \frac{\eta_{0.15MKCl}}{\eta_w} \cdot \frac{1 - \bar{V}\rho_{20,w}}{1 - \bar{V}\rho_{0.15MKCl}}$$

where

$S^{0}_{20,0.15MKCl}$ is the extrapolated value for the sedimentation coefficient in 0.15M potassium chloride,

$\frac{\eta_{0.15MKCl}}{\eta_w}$ is the relative viscosity of 0.15M potassium chloride with respect to water at 20°C.,

$\bar{V}$ is the partial specific volume of the solute,

$\rho_{0.15MKCl}$ is the density of 0.15M potassium chloride at 20°C,

ρ_w is the density of water at 20°C.

The density of water at 20°C was found in standard tables. The density of the solvent was estimated by a pycnometric method (Washburn and Smith, 1934). Two 25 ml. silica pycnometers were taken and their difference in weight estimated using a twin-pan semi-micro balance. One was filled with distilled water and the other with 0.15M potassium chloride and equilibrated at 20°C for thirty minutes and the difference in weight determined. This latter procedure was repeated, except that the contents of the pycnometers were reversed. During this procedure, each pycnometer was always placed on the same balance pan. In order to standardise the moisture adsorbed onto the pycnometers, the same procedure was adopted with each pycnometer. After filling with either water or potassium chloride cooled to approximately 15°C, the pycnometers were allowed to equilibrate in the accurately controlled water bath (used for the viscosity measurements) at 20°C for thirty minutes. The pycnometers were removed and carefully

wiped with tissues and allowed to equilibrate in the balance for twenty minutes before weighing. The result was calculated according to the formulae given by Washburn and Smith (1934).

$$D - D_w = \frac{(m_1 + m_2)}{V_1 + V_2}$$

Where D = density of 0.15M potassium chloride at 20°C

D_w = density of water at 20°C

m_1 = difference in masses after first filling

m_2 = difference in masses after second filling

$V_1 + V_2$ = total volume of the pycnometers.

The volumes of each pycnometer was determined by differential weighing with one empty and the other filled with distilled water at 20°C.

Ultracentrifuge studies

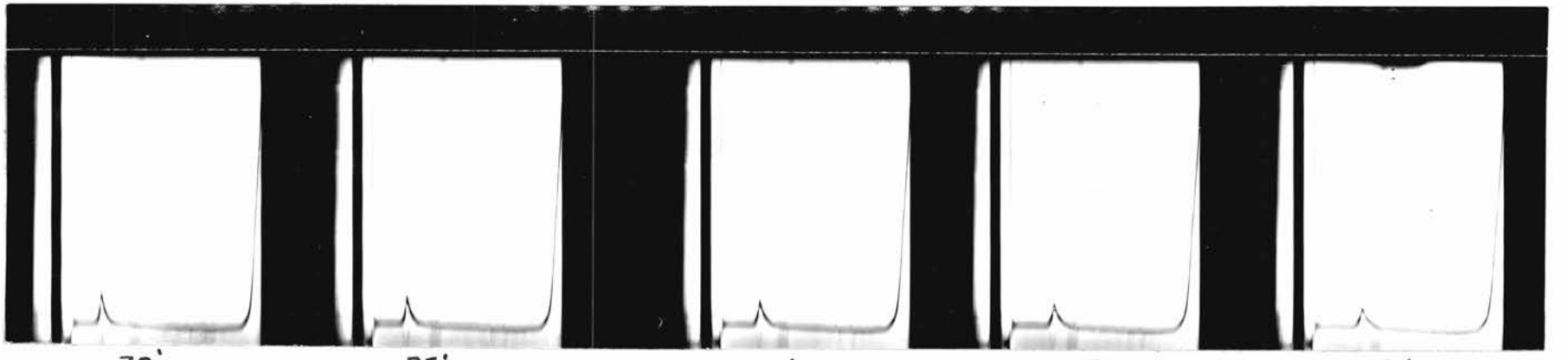
The 'runs' were performed in 3, 6 or 12 mm. cells, depending on the concentration used, at 59,780 r.p.m., in a Beckman Spinco Model E analytical ultracentrifuge. Photographs were taken every five minutes using the Schlieren optical system. All runs were performed at 20°C. The sedimentation coefficients were measured by the method of Elias (1963). After development, the distance of the sedimenting peak from the reference line

was measured with a Beck travelling microscope. Thus the distance peak to centre of rotation was calculated by adding this figure to the value for reference line to centre of rotation, as supplied by the manufacturers. The distance from peak to reference line was measured in each photograph (X) and thus the movement (ΔX) of the peak in a certain time (Δt) could be calculated. The mean of the two values of X, separated by distance X was also calculated ($\bar{X}$). The sedimentation coefficient for each pair of photographs was determined using the formulae:-

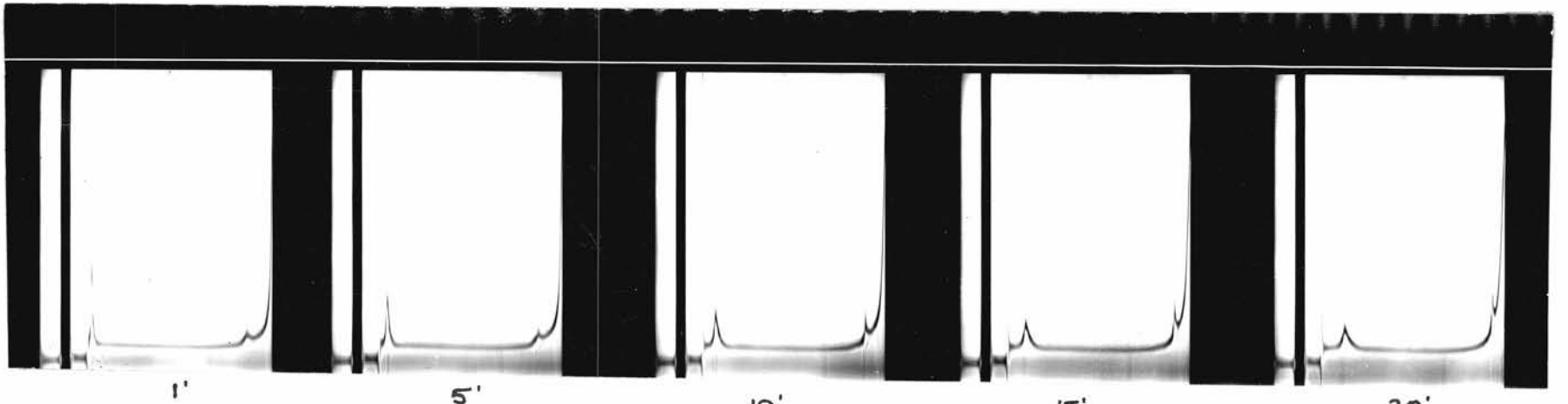
$$Sp = \frac{\Delta X}{\bar{X}\,\Delta t} \cdot \frac{1}{60w^2}$$

where $$w = \frac{2\pi \times r.p.m.}{60}$$

The sedimentation coefficient for that particular concentration was the mean of all the values for Sp omitting any obviously divergent readings. The figures obtained from a typical run are shown in Fig. XVI.



ULTRACENTRIFUGE RUN OF BISMUTH PURIFIED PP-L (4.5 mg/ml) 59,780 r.p.m.
SCHLIEREN OPTICS 60° TEMP 20°C. PHOTOGRAPHS TAKEN AT INDICATED TIMES



ULTRACENTRIFUGE RUN OF CPC PURIFIED PP-L (6.24 mg/ml) 59,780 r.p.m.
SCHLIEREN OPTICS 60° TEMP. 20°C. PHOTOGRAPHS TAKEN AT INDICATED TIMES

Fig. XVI

Solution 0.624% CPC purified PP-L

Distance meniscus to reference line - 0.1883 cm.

meniscus to centre of rotation - 5.8883 cm.

$w^2 60$ (59,780 r.p.m.) - 2.39×10^9

t	X	ΔX	$\bar{X}$	Δt	Sp
1	5.917	0.027	5.931	4	4.84
5	5.944	0.034	5.961	5	4.85
10	5.978	0.034	5.995	5	4.82
15	6.012	0.034	6.029	5	4.79
20	6.046	0.037	6.065	5	5.19
25	6.083	0.037	6.102	5	5.16
30	6.120	0.035	6.137	5	4.85
35	6.155	0.035	6.173	5	4.82
40	6.190	0.037	6.209	5	5.07
				Average value	4.93

With some of the more dilute solutions of the proteoglycans it was necessary to take pictures more frequently than every five minutes due to the rapid collapse of the peak at this concentration. The S^{o}_{20}, 0.15M KCl was estimated for each compound by plotting S^{c}_{20} against concentration and extrapolating to zero. An alternate method of calculation was to plot $S^{c}_{20} \cdot \frac{h^c}{\eta^o}$ against concentration

where $\eta c/\eta o$ is the relative viscosity of the solution whose sedimentation coefficient had been determined. The value of $S^c_{20}. \eta c/\eta o$ at a concentration near to zero is equal to S^o_{20} as $\eta c/\eta o$ equals 1 at zero concentration. Table XVII gives the results obtained using the bismuth nitrate purified PP-L.

Table XVII

Concentration (gm./100 ml.)	$\eta c/\eta o$	$S^c_{20}. \frac{\eta c}{\eta o}$	S^c_{20}, 0.15M KCl.
0.901	-	-	2.01
0.680	-	-	2.26
0.45	3.429	20.2	5.52
0.405	2.939	19.0	-
0.365	2.618	18.3	-
0.330	2.368	17.6	-
0.296	2.149	16.9	-
0.230	-	-	9.38
0.11	-	-	11.86

Where the sedimentation coefficient had not been measured at the same concentration at which the viscosity

measurements were carried out, a value for the sedimentation coeffficient was read from the graph of S^c_{20} against concentration. From the figures given in Table XVII, the value for the S^o_{20}, 0.15M KCl calculated are as follows:-

S^o_{20}, 0.15M KCl extrapolated from S^c_{20} values - 11.71 Svedberg units.

S^o_{20}, 0.15M KCl extrapolated from $S^c_{20}.\frac{\eta c}{\eta o}$ values - 10.82 Svedberg units.

The mean of these two values was corrected to give a value for $S^o_{20,w}$ using the formula given on page . The method of calculating S^o_{20} values by extrapolating a graph of $S^c_{20}.\frac{\eta c}{\eta o}$ against c is that given by Schachman (1959) and although it has not been devised from basic thermodynamic principles, it has been shown empirically to be of value.

Fluorescence

A sample of PP-L was examined in a Fluorispec SF - 1 spectrophotofluorimeter and found to have a fluorescent maximum at 350 mμ for an excitation maximum of 290 mμ. Fig. XVIII shows a graph of spectrophotofluorimeter readings for $E_{290} \rightarrow E_{350}$ against concentration for a sample of PP-L. The explanation for this fluorescence is not clear. The excitation and fluorescence

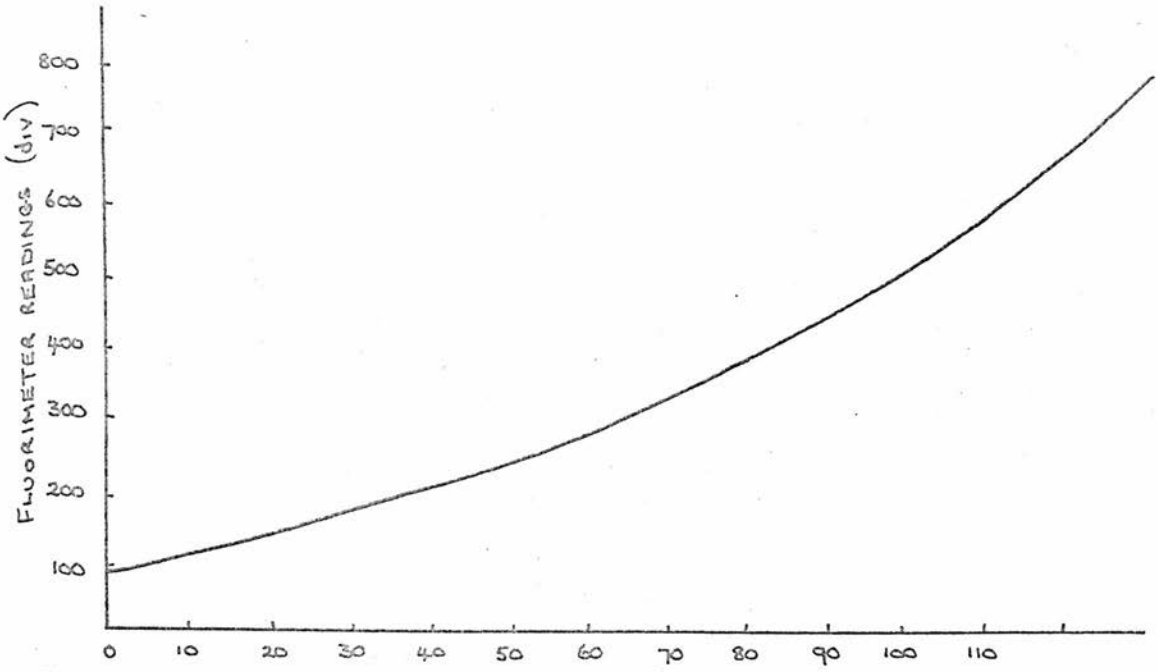


FIG XVIII

GRAPH OF FLUORESCENCE OF PP-L IN 0·15 M POTASSIUM CHLORIDE EXCITATION 290 mµ FLUORESCENCE 348 mµ

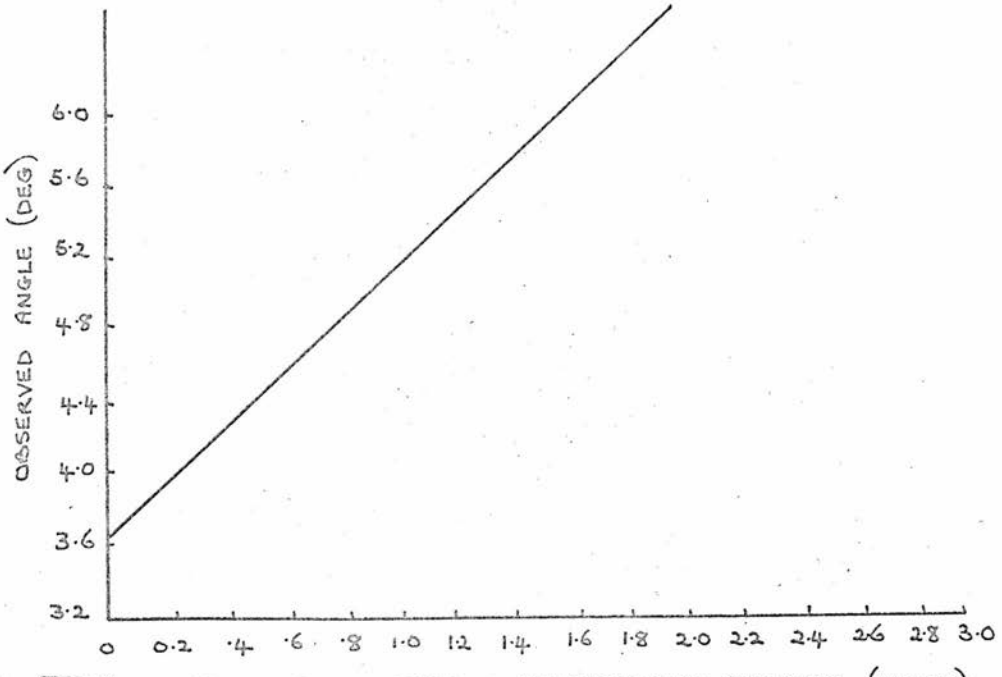


FIG XVIX

CALIBRATION CURVE FOR ESTIMATION OF POTASSIUM CHLORIDE BY REFRACTOMETRY

maxima of the PP-L correspond to that of tryptophan (Teale and Weber, 1957). However, no significant amounts of tryptophan are detectable in the proteoglycan. The complex has been shown to be capable of absorbing ultraviolet light (Gerber, Franklin and Schubert, 1960) but the relative contributions of the polysaccharide and protein moieties to the fluorescence are not known.

Ultraviolet Spectra

The spectra of samples of proteoglycans were recorded automatically in 1 cm. silica cells, using a Unicam SP800 spectrophotometer. This instrument was also used to plot the change in optical density at constant wavelength during the estimation of thiol groups and whilst following the effect of alkali on samples of the proteoglycan.

Infra-Red Spectra

Samples of the various preparations of PP-L which were studied, and samples of hyaluronic acid and chondroitin 4-sulphate which were used in preliminary experiments, were ground to a fine powder in an agate capsule using a vibration mill. All samples were examined in Nujol mulls, in a Unicam SP200 G grating infra-red spectrophotometer. The facilities were not available for the preparation of potassium bromide discs.

Mulls prepared using hexachlor-1,3,-butadiene, instead of Nujol, were used to examine the spectra of the polysaccharide in the regions where the Nujol gave absorption maxima.

Electron Microscopy.

Homogenised samples of the stained material were either sprayed or floated onto carbon filmed grids and viewed in a Siemens Elmiskop I electron microscope. Initially, the samples of PP-L were stained using an aqueous solution of bismuth nitrate in nitric acid (Serafini-Fracassini and Smith, 1966). 1 gm. of bismuth nitrate (A.R. grade) was dissolved in 10 ml. of 2M nitric acid. To this, 190 ml. of water were added whilst the mixture was being stirred mechanically. The pH of this solution was 1.9. Equal volumes of this solution of bismuth nitrate and squeous solutions of PP-L were mixed and allowed to stand for 15 minutes at room temperature. The mixture was centrifuged for 10 minutes at 3,000 r.p.m. and the residue washed once with 0.01M nitric acid and three times with water. The residue was suspended in a small volume of water and sprayed onto the grids. If the residue was fibrous and of large particle size, more satisfactory results

were obtained if the residue was briefly homogenised in a Potter homogeniser, before spraying.

Serafini-Fracassini and Smith (1966) showed that *in vivo* there was a specific binding between collagen fibres and PP-L particles. Rows of particles were shown to be arranged along the a and b_1 bands of the collagen fibre at right angles to the main axis of the fibre. Some experiments were carried out to attempt to reconstitute this relationship, *in vitro*. Samples of PP-L (prepared by the method of Gerber, Franklin and Schubert, 1960) and acid-soluble calf skin collagen (prepared according to the method of Steven and Tristram, 1962) were mixed in various proportions at room temperature. A gelatinous precipitate was formed and attempts were made to stain this with the aqueous solution of bismuth nitrate by the method described above, but it was found that the precipitated collagen was immediately solubilised by the nitric acid present. The next stage was to precipitate the 'collagen - PP-L complex' from aqueous solution with acetone and to stain the precipitate with aqueous bismuth nitrate but again it was found that the collagen was solubilised. In fact, Heidemann (1964) has shown that acetone precipitation of acid-soluble collagen increases its subsequent acid solubility.

It was decided to try and combine the acetone and bismuth nitrate in a single reagent. This was prepared as above except that 190 ml. of A.R. grade acetone were added to the bismuth nitrate instead of the 190 ml. of water. The addition must be performed slowly to avoid the precipitation of bismuth sub-nitrate which tends to occur. Similarly for this reason it is not possible to prepare a solution of bismuth nitrate in concentrations of acetone other than 85-95%. The samples of proteoglycan with or without tropocollagen were 'stained' with bismuth nitrate in acetone, by slowly adding the solution to 3-5 vols. of bismuth nitrate in acetone, washing the residue with acetone once, and with water thrice. Some precipitation of bismuth subnitrate occurs, along with the stained polyanion but this gives rise to little confusion. The results of the application of these two staining techniques are discussed later in the Results Section.

Refractometry

One of the methods of purification of PP-L to be described later, consists of precipitating the proteoglycan-cetyl pyridinium chloride (CPC) complex in a cellulose column and eluting the material with a salt gradient. In

order to confirm that the system used did in fact produce a linear gradient, 'dummy runs' were performed in which the potassium chloride gradient was perfused through the washed column. Fractions were collected in the same manner as in a preparative run. Full details of the apparatus will be described in a later section. In this section, the method of estimating potassium chloride concentrations by refractometry will be described.

An Abbé High Accuracy Refractometer (Bellingham and Stanley Ltd.) was used. Light was used from a sodium lamp and the instrument was calibrated with the ordinary ray from the standard quartz slab using the value for refractive index of the quartz, provided by the manufacturers. As the critical angle of a solution alters by 30 seconds per C. degree change, at room temperature, the refractometer and all solutions to be assayed were allowed to stand in the balance room overnight before taking readings. The thermometer inserted in the prism box was read several times during the experiment and was found to vary by less than 0.5 C. degree. The instrument was calibrated by measuring the critical angle of a series of solutions of potassium chloride. The observed critical angle could be converted to refractive index using the tables supplied by the manufacturers but this is unnecessary for present

purposes and Fig. XVIX shows the calibration curve obtained. The fractions from the 'dummy run' were assayed and the concentration of potassium chloride in each fraction calculated from the calibration curve. Fig. XX is the elution chromatogram from such a 'dummy run' which produced a linear gradient between 1.0M and 2.5M potassium chloride. It is reasonable to presume that gradients in which different concentrations of potassium chloride or of magnesium chloride were used, would be linear but having a different slope.

Electrophoretic Esperiments

It was decided to compare the electrophoretic mobility of the various preparations of the proteoglycan PP-L. Various electrophoresis systems were tried before satisfactory results were obtained with a reasonably simple procedure. Although good separation and the quantitative assay of mixtures of acid mucopolysaccharides is readily obtained, e.g. Manley (1965), paper electrophoretic separation of proteinpolysaccharide components does not give discrete bands. Most of the published systems give either considerable tailing or the complex fails to move from the origin, e.g. Lowther and Baxter (1966) and Partridge (1966).

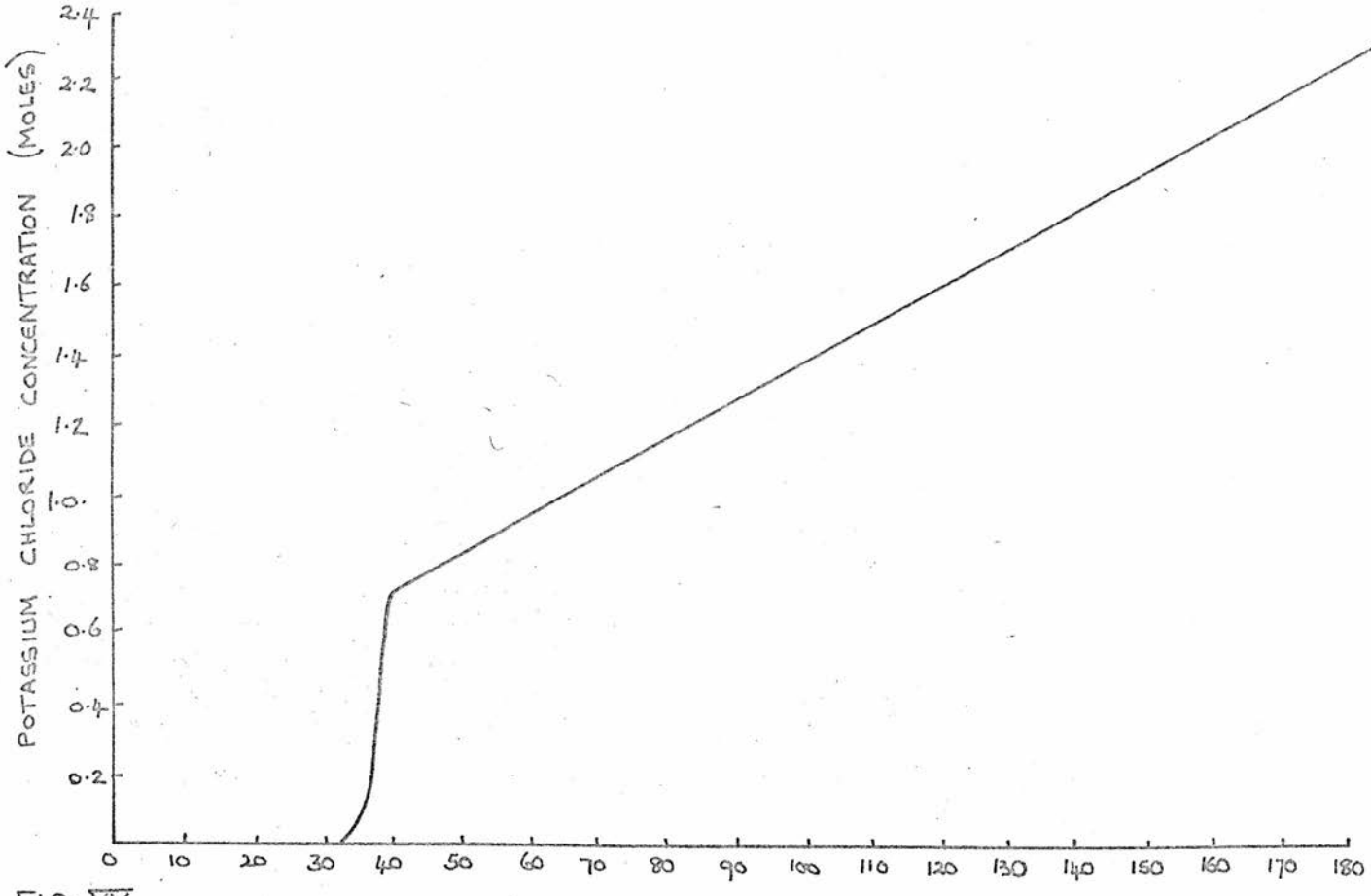


FIG XX

TYPICAL ELUTION CHROMATOGRAM FROM 'DUMMY RUN' PRODUCING LINEAR GRADIENT BETWEEN 0·8 AND 2·5 M POTASSIUM CHLORIDE

High voltage electrophoresis on various supporting media and in various buffers was tried. Electrophoresis in starch gel and on cellulose acetate and on paper were tried using McIlwain's phosphate-citrate buffer, (I=0.1), and in barbiturate buffers over the pH range 6 - 9 at potential gradients of up to 70 volts $cm.^{-1}$. The strips were stained with 1% Alcian blue in 5% acetic acid for thirty minutes and washed with 5% acetic acid and running water. Attempts to stain the protein moiety with 0.1% nigrosin in 5% acetic acid were unsuccessful. Using the high voltage system considerable streaking of the band occurred though this was found to be less at lower potential gradients.

The system giving the best results was as follows:- 30 x 3 cm. cellulose acetate strips were allowed to equilibrate overnight in a horizontal electrophoresis tank (E.E.L.). The electrolyte used was 0.1M lithium acetate. 5-20µl. of 1% solutions of the proteoglycans were applied to the strips and the current passed for 5 hours. The potential gradient was approximately 4 volts $cm.^{-1}$, and the current passed was 5 m.a., if three cellulose acetate strips were run simultaneously. The strips were dried and stained with 1% Alcian blue. Photographs of stained strips are shown in the Results Section.

C. Experimental Methods

Preparation of Hyaluronic Acid

Samples of this mucopolysaccharide were prepared for use in preliminary studies by electrophoresis, infra-red spectrophotometry and cetyl pyridinium chloride precipitation. The method used was that of Jeanloz (1965), using umbilical cords as a source.

Human umbilical cords were collected into a jar of acetone and stored at -15°C until processed. Approximately 100 gm. of dried cords were freed from blood and cut in small pieces. These were washed once with three litres of acetone, twice with distilled water for three hours and finally with water for twenty-four hours. The cords were ground into a paste with a mincing machine and three gm. of 'Difco' pepsin added, after adjusting the pH to 2.0. The mixture was stirred at 35°C gently for twenty-four hours in a warmed cabinet, after adding toluene to inhibit bacterial growth. The pH was adjusted to 7.5 with sodium hydroxide and 4 gm. of 'Difco' trypsin added and the mixture incubated for a further twenty-four hours. The mixture was cooled to 4°C and the pH adjusted to 2.0 with hydrochloric acid and two volumes of 99% ethanol added. The precipitate formed was collected by

centrifguation, suspended in one litre of water and dialysed for 24 hours against running tap water. To the contents of the dialysis sac were added, one litre of a solution containing 160 gm. of acetic acid and 300 gm. of sodium acetate. This solution was shaken vigorously with 800 ml. of chloroform and 400 ml. of 1-pentanol to denature any protein present. The mixture was centrifuged and the supernatant removed and retreated with chloroform : 1-pentanol. To the aqueous solution of hyaluronic acid, two volumes of 99% ethanol were added and the precipitate dialysed against tap water as before. The contents of the dialysis sac were diluted to 500 ml. and 500 gm. of ammonium sulphate added. 50 ml. of pyridine (A.R.) were added to the solution which was vigorously stirred. This mixture was kept overnight in the cold room and the precipitate collected by centrifugation, washed with ethanol and dialysed against several changes of distilled water and lyophilised. The yield of 1.5 gm. is less than that obtained in the original method. The purity of the product was confirmed by demonstrating a nitrogen:hexosamine:uronic acid, molar ratio of 1.0 : 1.0 : 1.0.

Preparation of bovine nasal cartilage chondromucoprotein and fractionation to give PP-L and PP-H.

The chondromucoprotein was prepared according to the method of Malawista and Schubert (1958) and this was fractionated by ultracentrifguation according to the procedure of Gerber, Franklin and Schubert (1960). Fresh bovine nasal cartilage was frozen overnight and planed into shavings with an ordinary wood worker's plane. 10 gm. aliquots of the shavings were homogenised in a large Waring Blender. The homogenisation was carried out in a cold room and was performed for a total of 20 minutes in 30 second sessions. The temperature of the extract was never allowed to rise above 15°C. Two volumes of chilled 99% ethanol were added to the extract and the mixture centrifuged at 2000 r.p.m. for 30 minutes in an M.S.E. 6L Mistral centrifuge. The supernatant was filtered through glass wool and 10 gm. of potassium acetate added to the filtrate which was allowed to stand overnight in the cold room. The residue was not re-extracted. The precipitate of the potassium salt of the chondromucoprotein was collected by centrifugation as above and washed twice with 99% ethanol and stored in a

frozen state. Fractionation of the chondromucoprotein into PP-L and PP-H was performed as soon as possible, the chondromucoprotein being kept in the frozen state meantime. 7.0 gm. of the crude chondromucoprotein were dissolved in 600 ml. of 0.15M potassium chloride by stirring mechanically in the cold room for 30-60 minutes. The opalescent solution was centrifuged at 20,000 r.p.m. (59,000 G) for three hours in an MSE 'Super 40' high speed centrifuge. The supernatants were decanted off, leaving a gelatinous residue together with about 10% of the supernatant and the mixture allowed to stand overnight. The residues were not re-dissolved in the potassium chloride and re-centrifuged to yield a further quantity of PP-L as it was felt that this would lead to a more heterogenous and degraded product. The precipitate of the potassium salt of PP-L was collected by centrifugation, washed with ethanol and dried in the frozen state. It was kept *in vacuo* until required. This procedure was also carried out on a sample of bovine articular cartilage to yield an analogous product.

Preparation of chondroitin 4-sulphate

A sample of PP-L was degraded with alkali to yield chondroitin 4-sulphate according to the method of Malawista

and Schubert (1958). 1 gm. of PP-L was dissolved in 75 ml. of 0.18M sodium hydroxide and allowed to stand at 37°C for 16 hours. 0.85 ml. of glacial acetic acid was added which altered the pH of the solution to 5.6. The mixture was dialysed against two changes of distilled water for 48 hours in the cold room. 1 gm. of barium chloride was added and ethanol slowly added to the constantly stirred mixture to give a final concentration of 20%. The solution was centrifuged for thirty minutes at 3,000 r.p.m. and the precipitate discarded. Ethanol was slowly added to the supernatant until a concentration of 50% had been reached. The precipitate of the barium salt of chondroitin 4-sulphate was collected by centrifugation and was dissolved in 75 ml. of distilled water and then precipitated in the presence of barium chloride as before. The precipitate of purified chondroitin 4-sulphate was used in preliminary experiments on the separation of C.P.C. complexes of proteoglycans and in I.R. studies.

Purification of the proteoglycan PP-L by precipitation with Bismuth Nitrate in acetone.

This purification procedure is based on the staining technique previously described. The proteoglycan PP-L is precipitated from aqueous solution with a solution of

bismuth nitrate in 95% acetone. The precipitate is washed with water and stirred with potassium chloride solutions. The potassium ions displace the bismuth ions and solubilise the proteoglycan which is subsequently precipitated from the solution by the addition of two volumes of 99% ethanol. This procedure is repeated and the final solution clarified by ultracentrifugation. The final procedure adopted after several modifications was as follows:-

1 gm. of crude PP-L prepared as previously described was dissolved in 50 ml. of 0.15M potassium chloride by stirring for thirty minutes in the cold room. To the magnetically stirred mixture, 200 ml. of bismuth nitrate in acetone, prepared as described under the section on electron microscopy, were slowly added. A flocculent white precipitate was recovered by centrifugation at 2,500 r.p.m. for 30 minutes in an M.S.E. 4 L centrifuge at 4°C. The supernatant was studied for its peptide and amino acid content as will be described later. The residue was washed with water and centrifuged as above. The residue was stirred with 250 ml. of 0.25M potassium chloride for three hours at 4°C and then centrifuged as above. The supernatant was added to two volumes of 99%

ethanol and allowed to stand overnight at 4°C, whence a fine white precipitate of PP-L formed. The residue, which consisted mainly of bismuth sub-nitrate did not yield any proteoglycan on further extraction with potassium chloride - 0.25M potassium chloride was found to be the minimum concentration which solubilised the proteoglycan. If lower concentrations were used, two or more extractions were necessary to solubilise the proteoglycan. The precipitate formed by the addition of two volumes of ethanol to the 0.25M potassium chloride extract was collected by centrifugation as above. This precipitate was extracted with a higher concentration of potassium chloride with the idea of displacing any bismuth ions still present. It was found that concentrations of potassium chloride greater than 1.0M gave a precipitation of potassium chloride crystals when mixed with two volumes of 99% ethanol and left overnight at 4°C. Early experiments were performed using 0.75M potassium chloride as an extracting solution but it was found that two extractions were necessary and in the definitive experiments the residue was extracted for three hours with 250 ml. of 0.5M potassium chloride. This extract was centrifuged as above and two volumes of 99% ethanol added to the supernatant which was left at 4°C overnight.

The precipitate which formed was collected by centrifugation and washed with ethanol and dried *in vacuo*. This preparation contains appreciable amounts of bismuth which is removed by ultracentrifugation. The partially purified proteoglycan (usually about 1.5 gm.) is suspended in 50 ml. of 0.15M potassium chloride by stirring mechanically for 1 hour in the cold room to give a slightly viscous cloudy solution. This is ultracentrifuged for 15 minutes at 78,000 G in an M.S.E. Super 40 centrifuge. The clear supernatant is decanted off leaving a white residue. The supernatant is exhaustively dialysed against several changes of distilled water in the cold room and the proteoglycan collected by lyophilisation and stored in a vacuum desiccator. From 1 gm. portions of crude PP-L, 0.7 to 0.85 gm. of the purified proteoglycan were obtained. Samples of this proteoglycan were assayed for ash, amino acid, moisture, hexosamine, nitrogen and protein content, by methods previously described. Samples of the purified proteoglycan were studied by I.R., U.V. and fluorescent spectroscopy. A portion was dinitrophenylated and the derivative formed studied by methods to be described later.

Steven and Tristram (1962) showed that if purified

calf skin acid-soluble collagen was precipitated by acetone at pH 3.5 or pH 11.0, amino acids and peptides originally associated with the collagen were found in the acetone supernatant. For this reason it was decided to examine the acetone supernatant from the bismuth nitrate in acetone precipitation, for amino acids and peptides, by thin layer chromatography (T.L.C.). It was found that the preparation of technically good chromatograms from the acetone supernatant which contains nitric acid and bismuth nitrate was exceedingly difficult. The procedure used was as follows:- The acetone supernatant was taken to dryness in a rotary film evaporator at 35°C. The residue was neutralised with sodium hydroxide and extracted twice with 50 ml. of n-butanol, previously equilibrated with N-hydrochloric acid. The butanol extracts were taken to dryness in a rotary film evaporator at 45°C and the residue, which still contained appreciable amounts of salt, was desalted using the resin Dowex 50 (Smith, 1960). A column of washed Dowex 50 x 8 in the H^+ form, 14 mm. x 15 cm. was prepared. The residue, dissolved in 5 ml. of water, was allowed to percolate through the column which was washed with 50 ml. of 4N ammonium hydroxide (A.R.). The amino acid solution was taken to dryness,

dissolved in a small volume of 80% acetone and subjected to thin layer chromatography. The developed plates were sprayed with both ninhydrin and the Morgan-Elson reagent to visualise amino acids and aminoglycans respectively. The technique of T.L.C. is described in a later section and the chromatograms discussed in the Results Section. The chromatograms were still technically rather unsatisfactory but further attempts at removal of the contaminating salt, for example, by use of further ion exchange resins, was not attempted as considerable losses of certain amino acids occur during this procedure. (Cook and Luscombe, 1960).

Purification of PP-L by gradient elution of the C.P.C. complex from a cellulose column.

Polyanions such as chondroitin 4-sulphate form highly insoluble complexes with cetyl pyridinium chloride (CPC) in aqueous solutions. The CPC-polyanion precipitate can be solubilised by salt solutions. The strength of salt necessary to solubilise the CPC-polyanion complex of a particular polyanion is highly reproducible (Critical Salt Concentration). As the charge density of the polyanion increases, the critical salt concentration increases (Scott, 1960). This procedure may be used in

the isolation, preparation and analysis of mixtures of different polyanions. The preliminary experiments followed the method of Scott (1960) but in the definitive method of preparation employed, certain modifications suggested by Antonopoulos et al.(1964) were adopted. The basis of the method used was to precipitate the CPC-proteoglycan complex onto a column of cellulose powder and then elute the column with a gradient of a salt solution. Because the salts of CPC are all insoluble at room temperature, it is necessary to use a jacketed column to keep the cellulose above the critical solution temperature (Scott, 1960). Thus, at room temperature (15 - 20°C) in the presence of a concentration of salt sufficient to dissociate a polyanion-CPC complex, the complex may not dissolve at all because the particles become coated with insoluble cetylpyridinium chloride which prevents further solvent action. On warming the mixture to 30-40°C, the complex readily dissolves. In the original method (Scott, 1960), the column was eluted with aqueous salt solutions but Antonopoulos et al. (1964) showed that unless a small excess of CPC was present in the system, elution artefacts occurred. Thus, these workers showed that shark chondroitin 4-sulphate gave a single peak when fractionated

with magnesium chloride containing 0.05% C.P.C. but three 'fractions' were obtained if aqueous magnesium chloride alone was used. These two important points were neglected in the fractionation of human aorta chondroitin 6-sulphate-protein complex by Buddecke and Schubert (1961). After precipitating the proteoglycan-C.P.C. complex onto the cellulose support, the column was washed first with 1% C.P.C. and then with 0.05% to ensure all the proteoglycan had been precipitated and, making use of the observation that the complex of kerato-sulphate and C.P.C. is soluble in excess C.P.C., to remove any free kerato-sulphate (Antonopoulos et al., 1964).

Early experiments were carried out using solutions of potassium chloride but in the definitive preparative technique, magnesium chloride in 0.05% C.P.C. was used as the eluate. Divalent cations allow a better discrimination during the elution than monovalent cations (Scott, 1960). Magnesium chloride is a very suitable salt because of its high solubility in ethanol. Fuller details of the method finally adopted are as follows:-

Apparatus

Fig. XXI is a flow diagram of the apparatus used.

The jacketed column (1.3 x 55 cm.) was fitted to a circulating pump and a 60 litre glass tank with heater, so that the contents of the column could be maintained at 30°C. The gradient vessels were two identical glass cylinders 6.5 x 20 cm. with a capacity of 600 ml. They were placed on level magnetic stirrers so that each vessel was stirred at 500 r.p.m. Sufficient heat was generated by the stirrers to warm the contents of the vessels so as to prevent precipitation of the magnesium salt of C.P.C. below the critical solution temperature. The ambient temperature of the room was at least 25°C during an experiment and the Teflon connecting tubes leading to the photocells were warmed in the circulating bath to 30°C. A D.C.L. micropump was used and throughout the experiments was set at 20 ml. per hour output. The effluent from the column was monitored with a Canalco Automatic Wide Track Recorder (Canal Industrial Corporation, U.S.A.). This instrument is very suitable for this type of experiment in which the eluting solution absorbs strongly in the U.V. region of the spectrum. By comparing the absorption of the eluant before and after passing through the column, the instruments can base compensate for any increase in solvent absorption of the eluate up to an 80% increase. This instrument

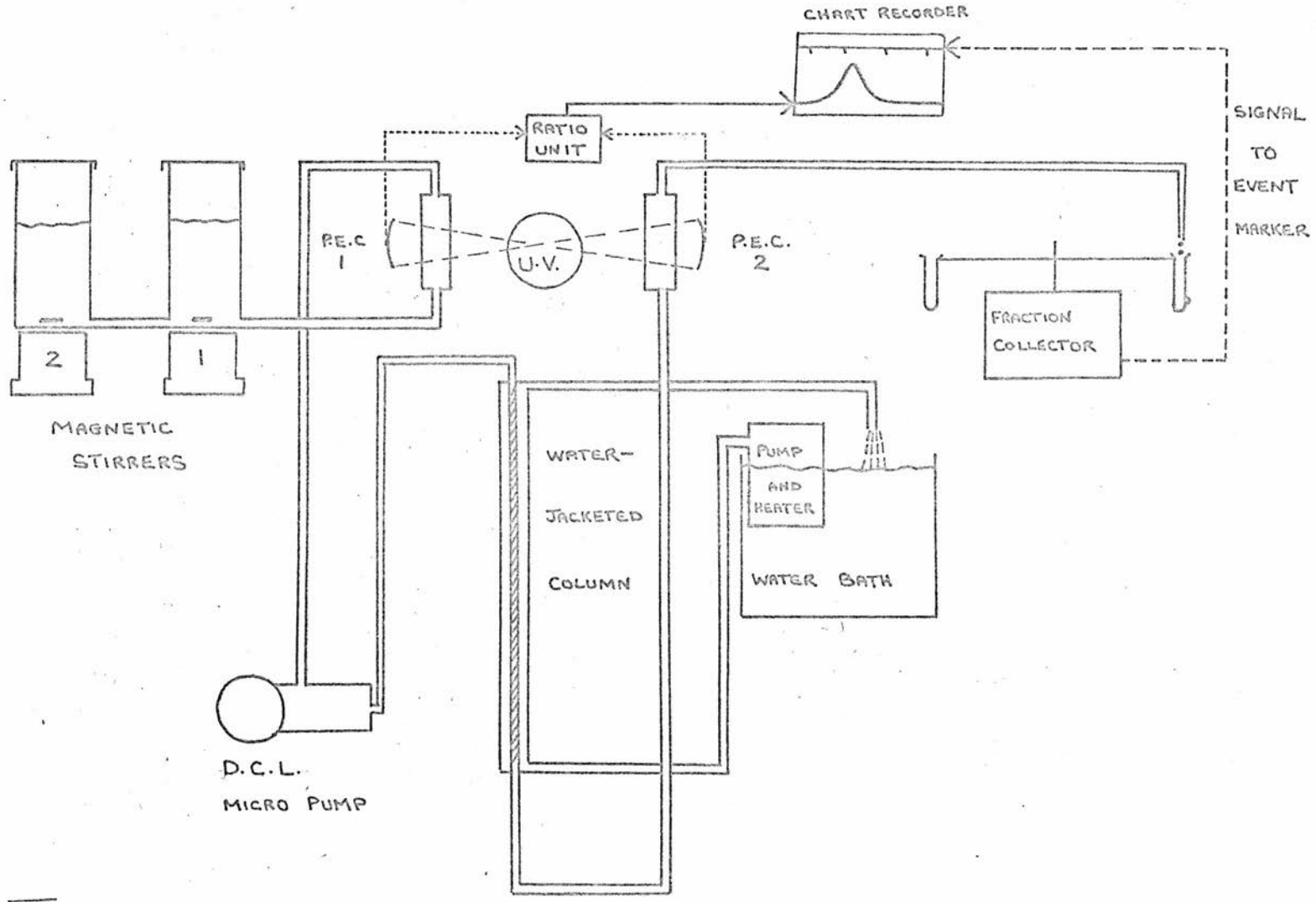


CHART RECORDER
RATIO UNIT
SIGNAL TO EVENT MARKER
P.E.C 1
U.V.
P.E.C. 2
FRACTION COLLECTOR
2
1
MAGNETIC STIRRERS
WATER-JACKETED COLUMN
PUMP AND HEATER
WATER BATH
D.C.L. MICRO PUMP

Fig XXI

is extremely sensitive and gives full scale deflection for difference in optical density between the contents of the flow cells of 0.18. When full scale deflection has been reached, the instrument automatically reduces its sensitivity by a factor of three so that the effective chart width is three times the actual width (7½"). Monochromatic light of wavelength 254 mμ is used and the silica flow cells have a capacity of 1 ml. with a light path of 20 mm. The chart speed was fixed at 4" per hour and an event marker activated by movements of the fraction collector was utilised. The effluent from the column, after monitoring, was collected using an L.K.B. Fraction Collector set to cut fractions at 30 minute intervals. Thus, with the flow rate of 20 ml./hour, fractions of approximately 10 ml. were obtained.

25-50 mg. of crude PP-L were dissolved in 3 ml. of 0.005M sodium sulphate by stirring in the cold room. The column was packed with a slurry of cellulose powder prepared as follows:- Approximately 100 gm. of cellulose powder (Whatman ordinary grade) were washed repeatedly by decantation with 5% acetic acid and distilled water. The 'fines' were removed by decantation. The slurry

was degassed by placing it in a stoppered Buchner flask and evacuating the flask with a water pump. The flask was gently rotated and warmed during this procedure. The column was packed by gravity and when a sufficient height of cellulose had been obtained the whole apparatus was washed by pumping distilled water through the system for 24 hours and with 1% cetyl-pyridinium chloride for 12 hours. The solution of PP-L was layered onto the top of the column and allowed to percolate into the cellulose powder. A small quantity of the cellulose slurry was added to the column after loading and the column washed with two bed volumes (70 ml.) of 1% CPC. to ensure precipitation of all the PP-L. 0.05% C.P.C. was pumped through the system overnight (16 hours) and the gradient connected. During the washing with aqueous solutions of C.P.C. the column effluent was monitored for change in ultra-violet absorption but fractions were not collected. The record obtained during the C.P.C. 'wash' showed a small peak emerging early in the wash. This was not analysed but is probably basic and neutral proteins which are not precipitated by C.P.C. and/or free kerato-sulphate which is soluble in excess C.P.C. (Scott, 1960). Buddecke and Schubert (1961) suggest

on the basis of its U.V. absorption spectra that this fraction consists of nucleopeptides and nucleic acids which contaminate their human aorta chondroitin 6-sulphate-protein complex. After the C.P.C. wash the gradient was connected and the fraction collector started. Various gradients of magnesium chloride and of potassium chloride were tried. All were linear but differed in slope and, initial and final salt concentrations. The system used in preparative experiments, which was found to give symmetrical peaks with a minimum of tailing, was prepared as follows:- 350 ml. of aqueous 0.05% C.P.C. was placed in the first chamber (1) and 350 ml. of 2M magnesium chloride in 0.05% C.P.C. placed in the second chamber (2) after removing any air bubbles in the connecting tube. Both chambers were stirred throughout the experiment because the C.P.C. tended to precipitate in the second vessel if the ambient fell much below 20°C.

The fractions were assayed for their polyanion content by the turbimetric method of Scott (1960). 2 ml. portions of each fraction were mixed with 3 ml. of 0.1% aqueous C.P.C. and briefly heated in a water bath at 30°C to solubilise any precipitated C.P.C. The turbidity was read against a water blank in 1 cm.

glass cells in a Unicam S.P.600 spectrophotometer at 400 mμ. Attempts to assay the fractions for their protein content by the Folin-Lowry procedure were unsuccessful, as the cetylpyridinium chloride itself reacts strongly with this reagent. A typical elution chromatogram is shown in the Results Section. The fractions corresponding to the peak were pooled and the proteoglycan collected. Most of the C.P.C. can be removed by cooling the fractions in an ice bath and removing the precipitated C.P.C. by filtration through a chilled, sintered glass funnel. Two volumes of 99% ethanol were added to the filtrate and the mixture allowed to stand overnight in the cold room. The precipitated proteoglycan was collected by centrifugation, washed with ethanol and dried *in vacuo*. A yield of approximately 75% was obtained. Samples of C.P.C. purified proteoglycan were assayed for their nitrogen, hexosamine, ash, moisture, protein and amino acid composition.

Studies on the proteolytic action of PP-L.

Partridge, Davis and Whiting (1965) showed that prolonged incubation of aqueous PP-L led to auto-degradation with breakdown of the protein core yielding

peptides and amino acids. It was suggested that this autodegradation was due to contaminating proteolytic enzymes and it was decided to investigate this suggestion further. Using bovine haemoglobin as a substrate, samples of freshly prepared PP-L were tested for any proteolytic activity in buffers of different pH. The method used was that of Davis and Smith (1955). 2.5 gm. of bovine haemoglobin (Sigma), which had previously been exhaustively dialysed against distilled water and lyophilised, were dissolved in 100 ml. McIlwain's citrate-phosphate buffer (I=0.1) pH 4.0 to which 2.0 ml. of 1/1000 thiomersal had been added and the solution warmed to 37°C. 0.5 ml. of a 1% solution of fresh PP-L was added and the contents of the flask maintained at 37°C in a metabolic shaker. At timed intervals after the addition of the proteoglycan 5 ml. aliquots were removed and quickly mixed with 10 ml. of 0.3M trichloracetic acid. After mixing, the contents of the tube were allowed to stand at 40°C for 30 minutes to ensure complete precipitation of the haemoglobin. The solutions were filtered and 5 ml. aliquots added to 10 ml. of 0.5N sodium hydroxide and 3 ml. of freshly diluted Folin-Ciocalteu reagent added.

The contents of the tubes were thoroughly mixed and read after 5 minutes in 1 cm. glass cells in a Unicam S.P.600 spectrophotometer at 650 mμ. A reagent blank and a standard solution of tyrosine containing 8×10^{-4} mEq/5 ml. were similarly treated. As this preliminary experiment had shown slight but definite evidence of proteolytic activity, the experiment was repeated in a modified form so as to measure the relative proteolytic activity of the proteoglycan over a range of pH. Several flasks were set up containing 2.5% haemoglobin in citrate-phosphate buffers over the range pH 2 - 8. 10 mg. of PP-L were added to each flask which was incubated as before for 1 hour, whence 5 ml. aliquots from each flask were assayed for tyrosine containing non-protein nitrogen. A graph showing the relative activity of the proteolysis at different hydrogen ion concentrations is shown in Fig. XXII. This shows two optima at pH 3.0 and 7.5 approximately. The preliminary experiment was repeated using larger quantities of PP-L in haemoglobin accurately buffered to these hydrogen ion concentrations. Fig. XXIII shows the release of tyrosine with time during the incubation at the two hydrogen ion concentrations. The results are discussed in the Results Section. No proteolytic activity could

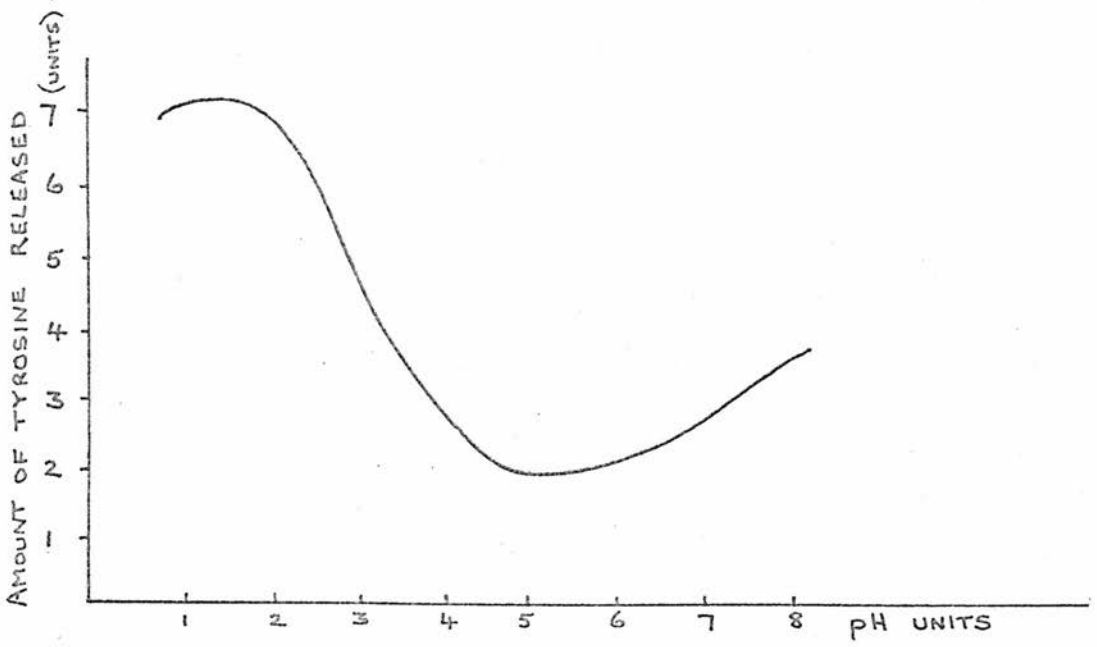


FIG XXII GRAPH SHOWING CHANGE IN PROTEOLYTIC ACTIVITY OF THE PROTEOGLYCAN WITH pH

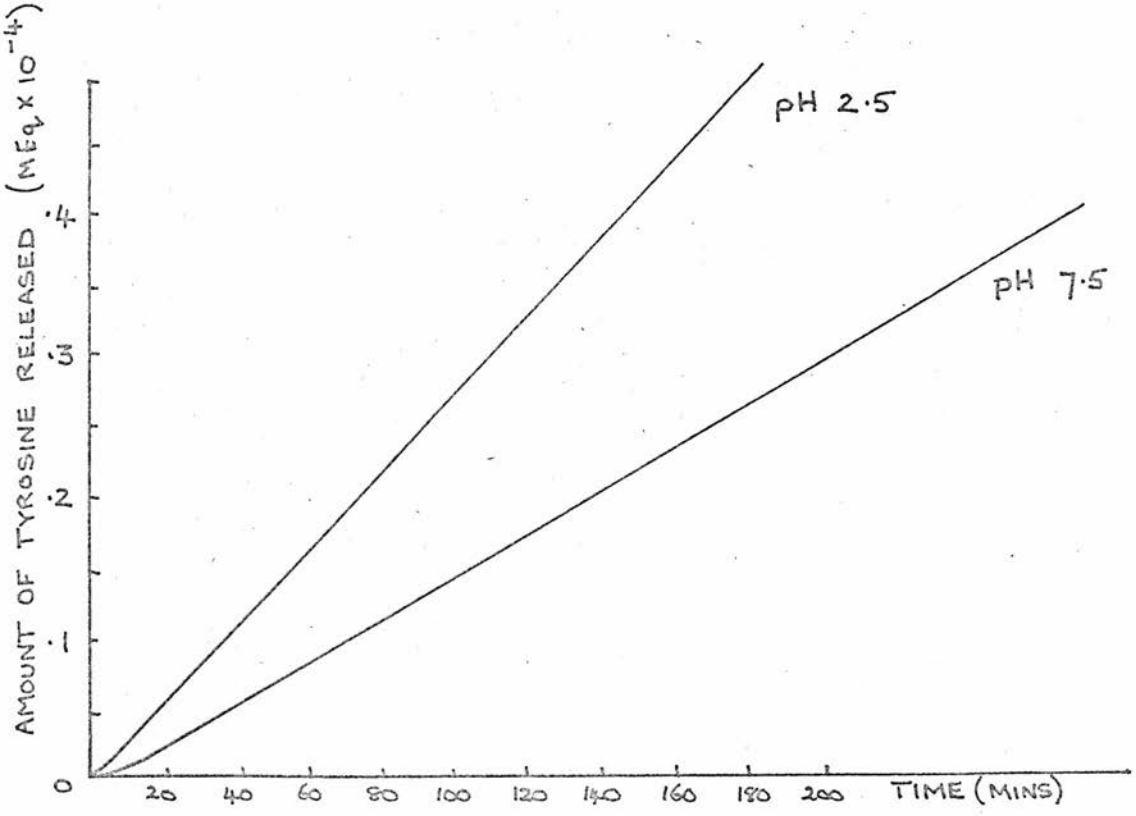


FIG XXIII GRAPH SHOWING RELEASE OF TYROSINE BY PP-L AT THE TWO pH OPTIMA

be demonstrated in the proteoglycan after purification by precipitation with bismuth nitrate in acetone or with cetylpyridinium chloride when tested at pH 3.5.

Effect of alkali on the proteoglycan PP-L.

As discussed previously, one of the effects of alkali on this compound is the cleavage of the O-glycosidic bond between serine and xylose. By a process of β elimination, serine is converted into α-amino-acrylic acid which absorbs strongly at 241 mμ. Using this observation, Neuberger, Gottschalk and Marshall (1966) have elegantly demonstrated the presence of O-glycosidic linkages to serine and threonine in glycopeptides prepared from ovine submaxillary gland mucin. It was decided to apply this technique to samples of crude and purified proteoglycan to ensure that the purification procedure had not cleaved this part of the molecule. Fig. XXIV shows the ultra-violet absorption spectra of a sample of PP-L before and after treatment with alkali; similar results were obtained with both bismuth nitrate and C.P.C. purified PP-L. The appearance of an inflection at 241 mμ during alkali treatment was followed automatically in a Unicam S.P.800 spectrophotometer. The instrument was set to

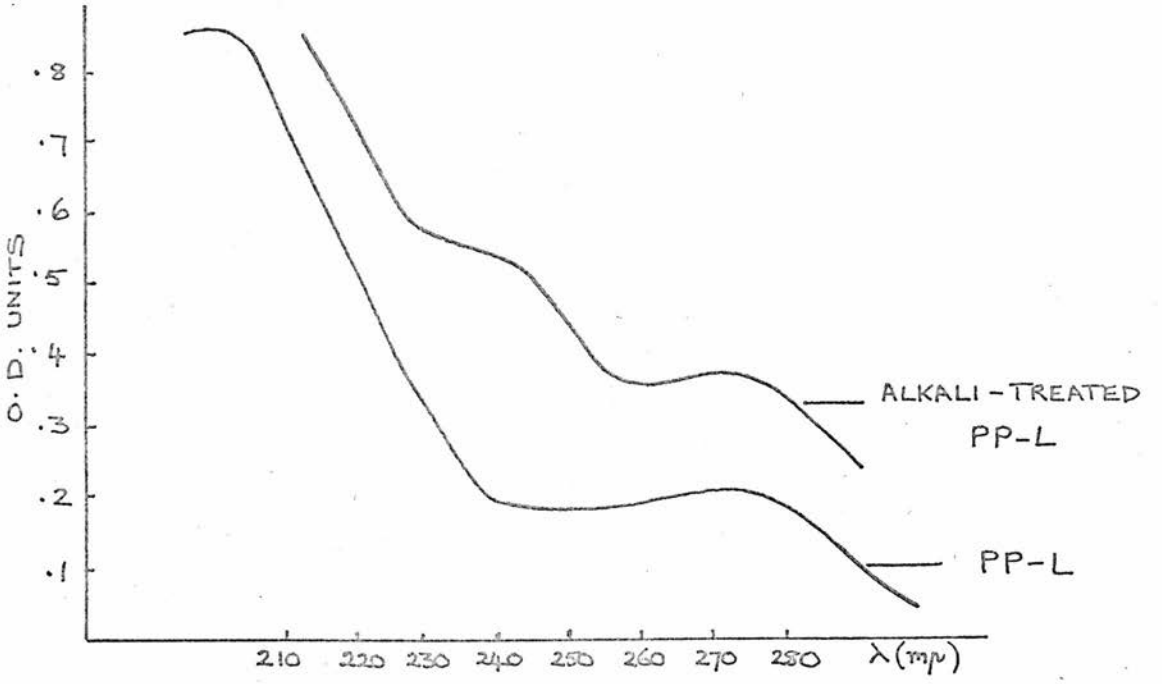


FIG XXIV ABSORPTION SPECTRA OF PP-L BEFORE AND AFTER ALKALI TREATMENT

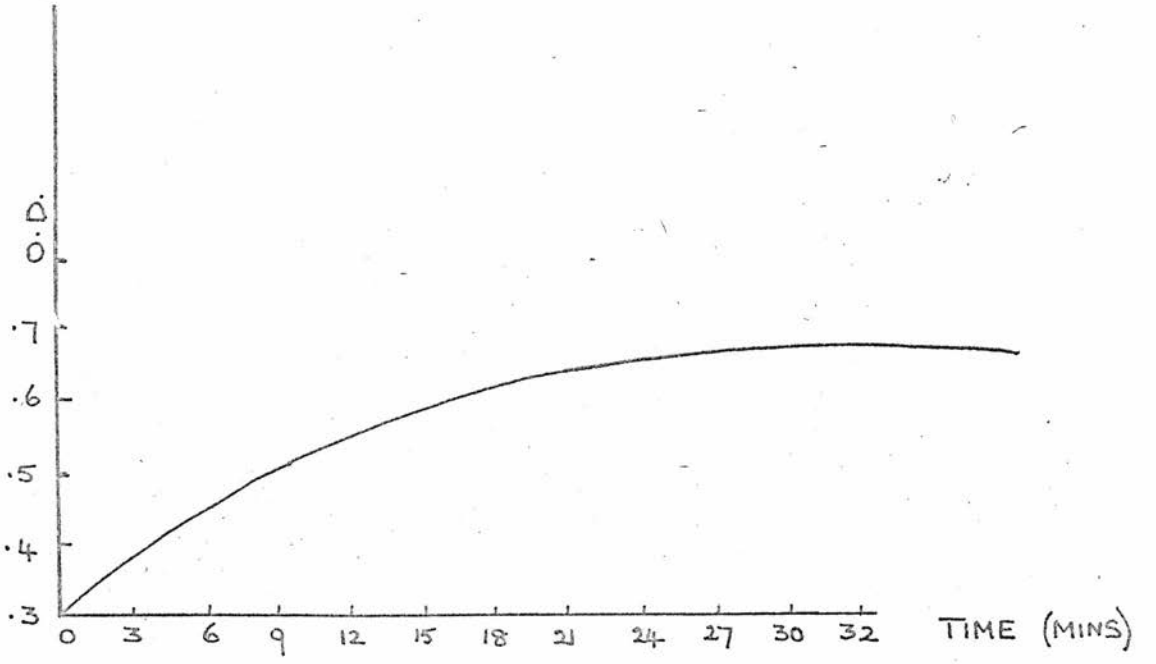


FIG XXV GRAPH SHOWING RISE IN E_{241} OF A SAMPLE OF PP-L TREATED WITH ALKALI

record changes in optical density at 241 mμ. Into a 1 cm. silica cell equal volumes of 1.0N sodium hydroxide and 1% solutions of the proteoglycans in 0.15M potassium chloride were added and the increase in optical density recorded over the next 30 minutes. A solution of 0.5% PP-L was used as a blank. Fig. XXV is a copy of such a graph obtained with a solution of crude PP-L. An identical rise in optical density occurred using 1% solution of the purified PP-L, indicating that a similar quantity of α-amino-acrylic acid was produced in each experiment. The absence of any inflection at 231 mμ in the alkali treated proteoglycans is evidence against the participation of threonine in O-glycosidic bonds to xylose (Neuberger, Gottschalk and Marshall, 1966).

Effect of Lithium Bromide and Hydroxylamine on the proteoglycan PP-L.

Early experiments on the structure of the macromolecule as shown by the electron microscope (Smith, Serafini-Fracassini and Peters, 1966) suggested that it consisted of rows of spherical particles. It was originally thought that PP-L may have been a polymer, each of the particles visualised representing a monomer.

Later studies using bismuth nitrate in acetone as a stain suggested that this view was incorrect (Serafini-Fracassini and Smith, 1966) but some preliminary experiments based on the earlier model were performed to attempt to depolymerise the macromolecule. Solutions of the crude proteoglycan were treated with 2M lithium-bromide and with 0.5M hydroxylamine and the effect of these reagents on the relative viscosity of the proteoglycan solution noted. The sedimentation coefficient of a sample of lithium-bromide treated PP-L was measured and samples of both types of modified PP-L were stained with bismuth nitrate and viewed with the electron microscope. Both the results and the experiments themselves will be discussed in this section because of their preliminary and inconclusive nature.

Lithium Bromide

2M-lithium-bromide is well known as a useful agent for splitting hydrogen bonds and it was thought that the PP-L 'monomers' may be joined by hydrogen bonds to form the macromolecular polymer. Equal volumes of 1% PP-L in McIlwain's buffer pH 7.0 and 4M-lithium-bromide were mixed in a capillary viscometer and serial measurements of the flow time made. Fig. XXVI is a

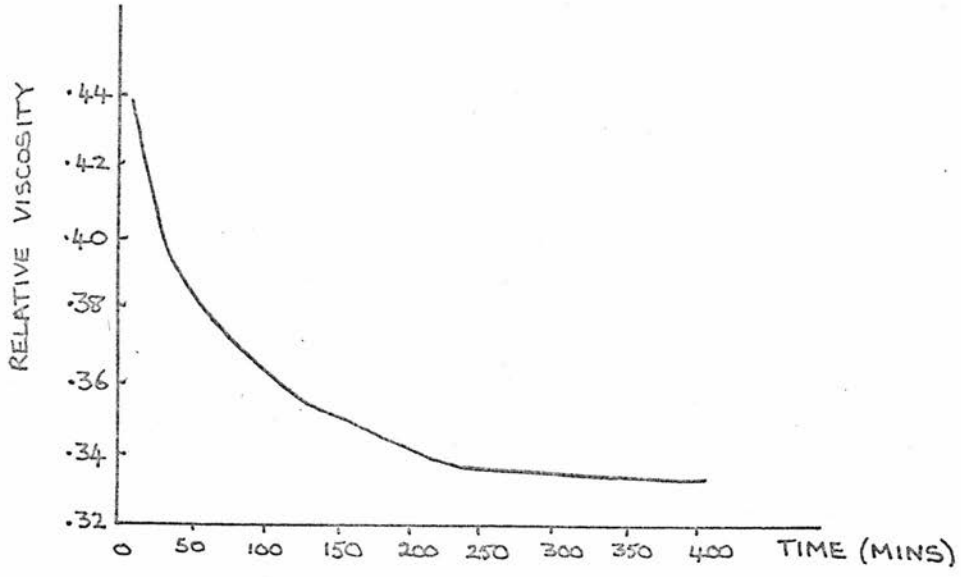


FIG XXVI GRAPH SHOWING FALL IN INTRINSIC VISCOSITY OF A 0·5% SOLUTION OF PP-L TREATED WITH 2 M LITHIUM BROMIDE AT 25°C

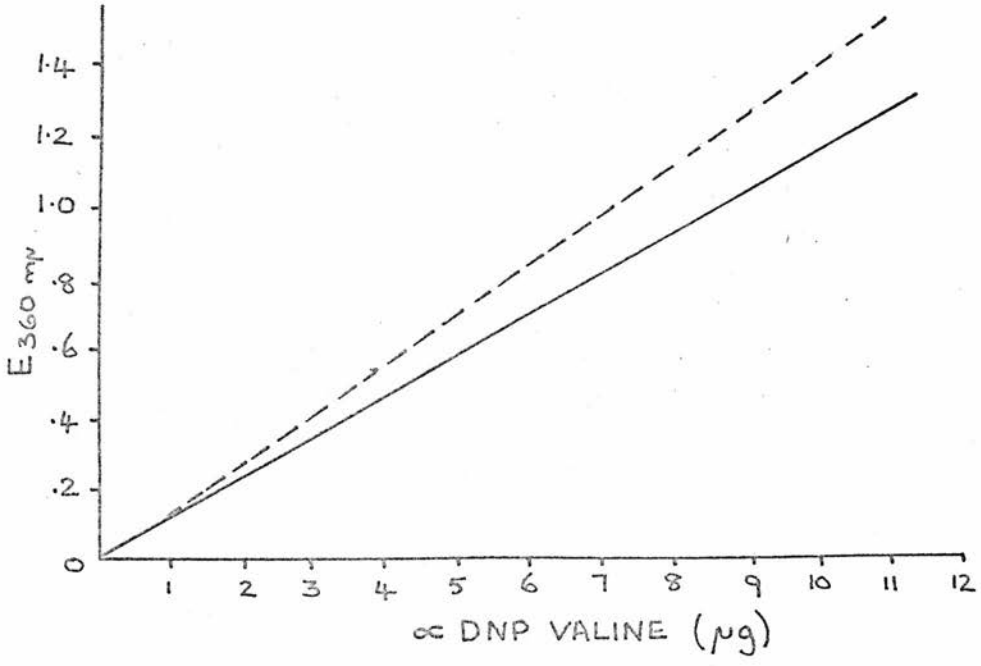


FIG XXVII CALIBRATION CURVE FOR ∝ DNP VALINE ESTIMATED DIRECTLY (- - - -) AND AFTER QUANTITATIVE T.L.C. (——)

graph showing the fall in relative viscosity,

$\frac{\eta - \eta_0}{\eta_0}$ with time

η = flow time of PP-L/lithium-bromide mixture (sec.)

η_0 = flow time of equal volumes of buffer and 4M-lithium-bromide.

The experiments were conducted at 25°C. A sample of PP-L similarly treated with 4M-potassium chloride showed no fall in viscosity. The lithium-bromide treated PP-L was exhaustively dialysed against 0.15M-potassium chloride and its volume adjusted so as to give a concentration of 0.5% proteoglycan. The dialysis was performed in a measuring cylinder at 4°C and was continued until no bromide was present, as detected using 1% aqueous auric chloride. Sedimentation coefficients for the untreated and lithium-bromide treated PP-L are given below; both migrated as single peaks.

$S^{o}_{20,w}$ crude PP-L 7.5s

$S^{o}_{20,w}$ lithium-bromide treated 6.1s

Samples of lithium-bromide treated PP-L were stained with bismuth nitrate and viewed with the electron microscope. No difference was noted between the treated

and untreated PP-L. There was no evidence that the particles were present in a separate and discrete form after treatment with lithium-bromide. No difference in the fluorescent spectra was noted between treated and untreated PP-L. The fall in viscosity and change in sedimentation coefficient following lithium-bromide treatment are probably due to a change in molecular configuration following cleavage of the hydrogen bonds which are partially responsible for maintaining the tertiary structure of the complex. It is unlikely that these minor changes in viscosity and sedimentation coefficient, taken in conjunction with the electron microscope findings, indicate that a depolymerisation of the macromolecule has occurred but rather a change in the molecular configuration.

Hydroxylamine

This reagent has been successfully used to demonstrate ester links in tropocollagen (Blumenfeld, Rojkind and Gallop, 1965). Studies on the use of this reagent on the proteoglycan PP-L have yielded negative results. Andersen, Hoffman and Meyer (1963), and Gregory, Laurent and Roden (1964) were unable to demonstrate the presence of hydrazides in the reaction

products following the action of hydroxylamine on PP-L. Pal and Schubert (1965) were unable to separate free chondroitin 4-sulphate following the action of hydroxylamine on PP-L. It was concluded from these studies that the carbohydrate-protein linkage of PP-L was not an ester bond.

A sample of PP-L was treated with 0.5M hydroxylamine hydrochloride at pH 9.5, prepared according to the method of Blumenfeld, Rojkind and Gallop (1965). 6.95 gm. of hydroxylamine hydrochloride were dissolved in 10 ml. of water and adjusted to pH 9.0 with 10N-sodium hydroxide. 10 ml. of 1M-potassium carbonate were added and the pH adjusted to 9.5. The mixture was made up to a volume of 50 ml. with water and kept in an ice bath until used. The reagent is unstable and must be prepared freshly for each experiment. The reaction was carried out at 25°C in a capillary viscometer as described previously. A marked and significant fall in viscosity was noted; a sample of PP-L treated similarly except that ammonium-chloride was used, instead of hydroxylamine hydrochloride, showed no change in viscosity. The reaction products were acidified and exhaustively dialysed against distilled water. The hydroxylamine treated PP-L

was stained with aqueous bismuth nitrate and viewed with the electron microscope and again no difference was noted between the treated and untreated PP-L. In the absence of ester bonds the explanation for the fall in viscosity on treatment of PP-L with hydroxylamine is not clear; a non-specific cleavage of the carbohydrate-protein link may have occurred.

The recent suggestion of Schubert (1966) that the chondroitin 4-sulphate chains are attached to polypeptides made up of 20 amino acid residues, which are themselves attached to the protein core at 50 amino acid residue intervals, has revived interest in the nature of the protein core. Schubert (1966) suggests that the polypeptide branches are cleaved by alkali from the central protein core but makes no suggestions as to the nature of the linkage. It may be in this region that the hydroxylamine is attacking the proteoglycan molecule without yielding hydrazide derivatives. Further tentative suggestions are made in the Results Section on the nature of this polypeptide branch point.

Thin Layer Chromatography

In this section the techniques used for the spreading and developing of chromatographic plates will be described. The spray reagents used, and the technique employed to keep

a permanent record of the chromatograms, will be considered. Quantitative thin layer chromatography (T.L.C.) was used to estimate the N-terminal amino acids of PP-L after dinitrophenylation. T.L.C. was used to investigate the amino acids and peptides in the acetone supernatant obtained during the purification of PP-L by precipitation with bismuth nitrate in acetone. This technique was also used to separate and identify the ether and water-soluble amino acid derivatives of hydrolysed, dinitrophenylated, PP-L. Chromatograms were also prepared of the amino acids released following hydrolysis of the dinitrophenylated proteoglycan.

Quantitative T.L.C.

The techniques used were essentially those described by Stahl (1965). Five 20 x 20 cm. glass plates were thoroughly cleaned with ethanol and distilled water and dried in an oven at 105°C. The cooled plates were placed in the spreading device (Shandon). A slurry of 30 gm. of Kieselguhr G. and 60 ml. of distilled water was rapidly prepared and the plates spread. They were dried by standing at room temperature overnight. Samples of the ether-soluble α-DNP-amino acid derivatives were spotted onto the prepared plates which were equilibrated overnight in a glass tank. Two-dimensional chromatograms

were run in all instances. Developing solvents for the α-DNP-amino acid derivatives were as follows:- A mixture of toluene-pyridine-ethylenechlorohydrin-0.8N ammonia (100:30:60:60) was prepared. The aqueous layer was removed using a separating funnel and was used in the overnight equilibration tank. The organic layer was filtered through several layers of filter paper to remove any water droplets and was used in the developing tank for the first dimension. After equilibrating overnight, the chromatogram was immediately developed in the first dimension. The solvent front was allowed to ascend for approximately 18 cm., being limited by a line drawn through the Kieselguhr parallel to the solvent front. The plate was dried in a dark cupboard in a stream of warm air. The plate was developed in the second dimension immediately. This solvent was prepared by mixing chloroform-benzyl alcohol-glacial acetic acid in the proportions, 70:30:3. Both dimensions were run in glass tanks, using solvents prepared weekly, which were placed in a light tight constant temperature cupboard at 55°F. After development in the second dimension the plates were dried as above. The plates were viewed under ultra-violet light to identify the DNP-amino acids. The

plates were either photographed as a permanent record or used for quantitative estimations. In order to obtain a permanent record of the chromatograms, the plates were placed upon Azoflex paper and were exposed to a bright light for approximately 30 seconds. The paper was immediately developed, using Azoflex 'Red' developer. This method is very sensitive and convenient and gives good records of the chromatograms obtained, particularly of DNP-derivatives.

The systems used for the separation of amino acids and water-soluble DNP-amino acid derivatives were as follows:- The first dimension was run in a solvent comprising n-butanol - glacial acetic acid - water (80:20:20, by volume). After development and drying in a current of warm air, the plate was developed in the second dimension. The solvent used was prepared as follows:- 75 gm. of phenol (A.R.) were warmed and dissolved in 25 ml. of distilled water. Approximately 100 mg. of Cupron were added as an anti-oxidant. This solvent was used in a chromatography cupboard at 65°F. After development, the plates were dried in a stream of warm air and photographed directly, to record any DNP-amino acid derivatives. The plates were then sprayed

to demonstrate the presence of amino acids, peptides and aminoglycans. Ninhydrin was used to detect the presence of amino acids. 0.3 gm. ninhydrin were dissolved in 100 ml. n-butanol and mixed with 3 ml. glacial acetic acid. After spraying, the plate was heated for 10 minutes at 110°C. The amino acids were identified by their R_f values, colour after reaction with ninhydrin, and rate of colour development. Before unequivocally deciding on the presence of a particular amino acid, a sample of the acetone supernatant or of the resin hydrolysis products of the dinitrophenylated PP-L was rehydrolysed with constant boiling point hydrochloric acid for 18 hours at 105°C in a sealed tube. The acid was removed in a rotary film evaporator at 35°C and the hydrolysate rechromatographed. This procedure is necessary when using resin hydrolysates because breakdown to the constituent amino acids may not be complete. Deane and Truter (1955) have demonstrated the identical behaviour of some peptides and amino acids on chromatography and thus it was necessary to employ this double hydrolysis procedure to confirm the presence of tyrosine in the absence of O-DNP-tyrosine in resin hydrolysates of dinitrophenylated PP-L, to be

described later. Peptides were more easily demonstrated after spraying with an alcoholic solution of ninhydrin, prepared as follows:- 0.1 gm. ninhydrin dissolved in 50 ml. absolute ethanol to which were added 10 ml. of glacial acetic acid. Aminoglycans were detected using the Morgan-Elson reaction. Spray I was prepared by mixing, immediately before use, 0.5 ml. of a mixture of 5 ml. of 50% aqueous potassium hydroxide and 20 ml. of ethanol, to a mixture comprising 0.5 ml. acetylacetone and 50 ml. n-butanol. The dried plate was sprayed with Spray I and heated to 105°C for five minutes and then sprayed with Spray II. Spray II was prepared by dissolving 1 gm. of dimethylaminobenzaldehyde in 30 ml. of ethanol and adding 30 ml. of concentrated hydrochloric acid. Immediately before use, this mixture was diluted with 180 ml. of n-butanol. After using Spray II, the plate was dried at 90°C, whence a cherry red colour appears in the presence of hexosamine. All chromatographic experiments were controlled by the use of internal and external standards.

Quantitative T.L.C.

In an attempt to quantitatively estimate the N-terminal amino acids of purified PP-L, the ether extract

of the hydrolysate was subjected to quantitative T.L.C. The extract was taken to dryness and made up to a known volume in acetone. Using a range of micropipettes, chromatograms were prepared with varying amounts of the mixture. A series of known amounts of mixtures of pure α-DNP-amino acids on chromatographic plates were also prepared. After two-dimensional chromatography, the plates were dried. The chromatograms were developed and dried in a light tight cupboard. All other procedures with DNP-derivatives were carried out under subdued lighting. The spots were removed from the plate, together with the surrounding 0.5 cm. of Kieselguhr, using the vacuum technique described by Ritter and Meyer (1962). The spots were sucked into paper 'thimbles' using the 'vacuum cleaner' described by these workers. The DNP-amino acids were slowly eluted with 5 ml. of chloroform/acetic acid (99/1). The yellow eluate from the thimbles, which were suspended over small test tubes, was read in a Unicam S.P.600 spectrophotometer using 1 cm. silica cells at 360 mμ against a chloroform/acetic acid blank. Elution of the thimbles with further solvent yielded no more U.V. absorbing material. It is important to

dry the plates thoroughly before the estimation to ensure the removal of the pyridine used in the first dimension development. Several 'blanks' were sucked off from different parts of the plate and the 'origin' was also similarly treated. All unknown solutions were estimated in quadruplicate and a standard curve was prepared for the main DNP-amino acids present. The content of the other DNP-amino acids were calculated using the value 11,500 as the molar extraction coefficient. Rao and Sober (1954) have shown that the extinction coefficients of the main α-DNP-amino acids with the exception of α-DNP-glycine, are very similar. Fig. XXVII is a standard curve for DNP-valine prepared directly from a standard solution, and following T.L.C. by the technique described above. The losses are constant at approximately 15%. This procedure offers several advantages over the usual paper chromatographic estimation of DNP-amino acids. The spots and origin are more discrete enabling better separation of the derivatives, e.g. DNP-aspartic and glutamic acids, to be obtained. Excluding the overnight equilibration the entire procedure can be completed within 5 hours. This technique is more sensitive than

the usual procedure using paper chromatography. Frankel-Conrat, Harris and Levy (1955) recommend the use of 0.1 - 0.2 μmoles of the DNP-derivatives, whilst using the present techniques 0.005 μmoles of α-DNP-amino acids can be estimated with reasonable accuracy. Thin layer chromatography suffers less from the problem of overloading and enables the separation of the DNP-leucine isomers to be achieved which is not possible using paper chromatography (Frankel-Conrat, Harris and Levy, 1955).

Dinitrophenylation of PP-L

Samples of crude proteoglycan and bismuth nitrate purified PP-L were dinitrophenylated using a pH Stat (Radiometer) to control the pH at which the dinitrophenylation was carried out. The autotitrator enabled the rate of reaction and the completion of the reaction to be determined. By calculating the amount of sodium hydroxide which had been utilised during dinitrophenylation, the number of reactive groups (mainly $-NH_2$) groups which had been substituted by fluorodinitrobenzene (FDNB) could be calculated. Dinitrophenylation was carried out at the following pH values: 6.5, 7.5 and 8.5, on samples of crude and purified PP-L. Experiments

were performed as follows:- Approximately 200 mg. of dried proteoglycan were dissolved, by stirring magnetically in the cold room, in 50 ml. of 0.15M-potassium chloride in a reaction vessel. The vessel was placed in the pH Stat which was set at the desired pH. The microsyringe was filled with 0.25N-sodium hydroxide and the reaction vessel connected to a cylinder of nitrogen which excludes carbon dioxide from the atmosphere above the reaction mixture. After the pH of the solution had been stabilised, 5 ml. of a freshly prepared 5% solution of fluorodinitrobenzene in absolute ethanol were added and the chart recorder started. The chart speed used was 25 cm. in eight and a third hours and the reaction vessel was enclosed in a dark cloth. The reaction was complete when the rate of titration had levelled off and was due solely to breakdown of fluorodinitrobenzene. Fig. XXVIII is a typical chart obtained during the dinitrophenylation of a sample of purified proteoglycan showing how the titration curve flattens off and indicates how the extrapolation back to zero time enables the amount of alkali taken up solely by the dinitrophenylation of the proteoglycan to be calculated. Although this method does not give an accurate value for the number

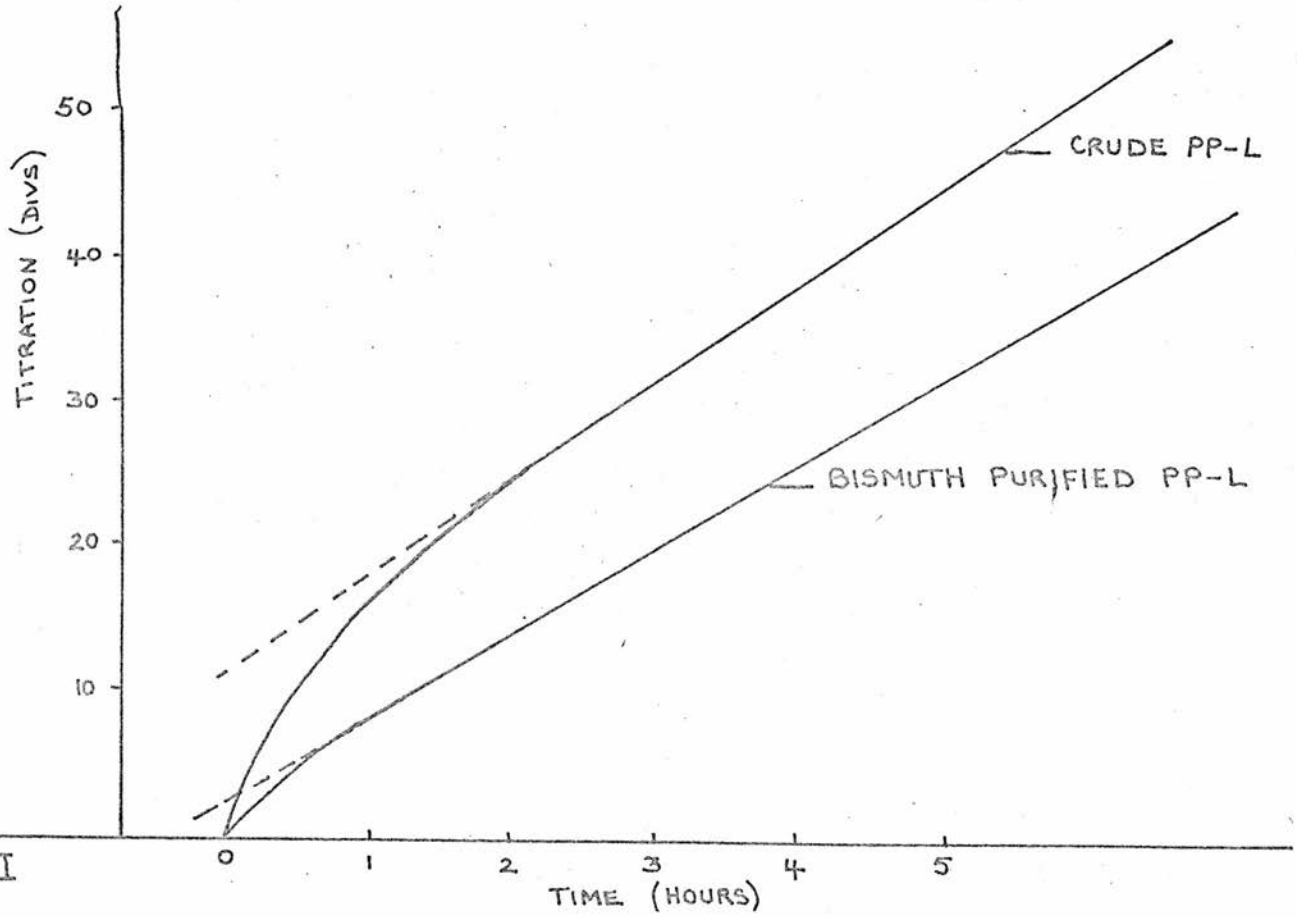


FIG XXVIII

TITRATION CURVE FOR SAMPLES OF CRUDE AND PURIFIED PP-L WITH FDNB IN A pH STAT AT pH 8·4 AT ROOM TEMPERATURE

of reactive $-NH_2$ groups, it does indicate the increase in reactive groups, as the pH of dinitrophenylation increased and shows a decrease in the number of reactive groups as the proteoglycan is purified. These results are discussed in the Results Section. After the reaction was complete, the viscid solution was exhaustively dialysed against distilled water in the cold room. The dinitrophenylated proteoglycan (DNP PP-L) was soluble even on acidification of the medium. Extraction of excess FDNB with ether was unsuccessful because of the high viscosity of the aqueous solution. Attempts to collect the DNP PP-L by precipitation with 10 volumes of acetone were also unsuccessful and the dialysed solution of DNP PP-L was lyophilised to yield a bright yellow derivative. This was stored in the dark, in vacuo, until further studied. Comparing the intensity of the dinitrophenylated derivative of the pure and crude proteoglycans, it was obvious that purification had removed many of the reactive groups. The ash and moisture contents of the derivatives were determined. The sedimentation coefficients and relative viscosity of samples of crude PP-L were estimated before and after dinitrophenylation. Table XXIX shows these results:

Table XXIX

	$S^{o}_{20,w}$	$\frac{\eta - \eta_o}{\eta_o}$ (0.5% solution)
Unpurified PP-L	7.7	3.2
Dinitrophenylated PP-L	4.6	12.1

These results are analogous to those obtained by Habeeb, Cassidy and Singer (1958) in their studies on succinylated proteins and are probably due to the unfolding or expansion of the molecule consequent upon the introduction of the dinitrophenol radicals.

N-terminal studies

Samples of crude and bismuth nitrate purified PP-L were dinitrophenylated as indicated in the previous section. The first attempt at hydrolysis was with 5.7N constant boiling point hydrochloric acid in a sealed tube at 105°C for 12 hours. Although the tube was thoroughly flushed out with nitrogen before sealing and there was a hundred-fold excess of doubly distilled hydrochloric acid, considerable humin formation occurred. Separation of the α-DNP-amino acids from the dinitrophenyl artefacts by the use of a silicic acid column

has been described by Steven (1962). An attempt was made to separate the α-DNP-amino acids from the humin artefacts by the use of this technique. The acid hydrolysate was diluted to approximately 100 ml. with distilled water and extracted five times with 50 ml. aliquots of diethyl ether. The extractions were carried out in a modified separating funnel which was fitted with a side arm and tap at the 100 ml. mark so that the repeated ether extracts could be separated directly. The combined ether extracts, which were washed once with 100 ml. of distilled water, obviously contained considerable amounts of brown humin derivatives. The water extract also contained a large amount of humin; attempts at chromatography on this water extract were completely unsuccessful due to gross streaking.

The ether extract was taken to dryness in a rotary film evaporator at 25°C and purified by silicic acid column chromatography by the method of Steven (1962a). 5 gm. of silicic acid (100 mesh) and 2.5 ml. of aqueous M/15-disodium-hydrogen-phosphate were mixed with a pestle and mortar and approximately 100 ml. of chloroform were added to give a fine slurry. This slurry was poured into a column (i.d. 9 mm.) to give

20 cm. of silicic acid. The ether extract was dissolved in a small volume of chloroform which had previously been equilibrated against M/15-disodium-hydrogen-phosphate and was carefully layered onto the top of the column. The column was washed with two 25 ml. portions of chloroform, previously equilibrated with M/15-disodium-hydrogen-phosphate, which eluted some of the humin derivatives as well as dinitrophenol and dinitroanaline. The DNP-amino acids were eluted with chloroform/glacial acetic acid (99/1). Although most of the humic acid remains bound to the top of the column, one orange-brown artefact band was eluted slightly ahead of the yellow DNP-amino acid band. The technique obviously separates most of the humin artefacts from the DNP-amino acids. However, considerable streaking occurred during two-dimensional chromatography of the eluted DNP-amino acids. The losses during this technique are high and variable (20-40%)(Steer, 1966: personal communication) and it was decided to rely on resin hydrolysis to release the DNP-amino acids. Satisfactory chromatograms of the ether extracts were obtained although small amounts of brown artefacts were obviously

present in the ether extract; these may be breakdown products of the resin itself. The resin hydrolysis was performed by a technique essentially that used by Steven (1962) in the hydrolysis of dinitrophenylated collagen. Dowex 50 x 8 resin was cycled through 2N-sodium hydroxide, distilled water and 2N-hydrochloric acid and a thick slurry of the resin in the H^+ form which had been washed free of hydrochloric acid was prepared. Hydrolysis of approximately 200 mg. aliquots were carried out in 30 ml. Pyrex culture tubes as used in the resin hydrolysis performed for estimation of uronic acids and hexosamines. At least 20 gm. of resin was used to ensure that no fluorodinitrobenzene reacts with any amino acids released during hydrolysis (Steven, 1962). The Pyrex tube was flushed out with nitrogen and hydrolysed at 100°C in an oven fitted with a device which enabled the tubes to be turned end over end at 10 r.p.m. Hydrolysis was carried out for 30 hours and after cooling, the resin was poured into a sintered glass funnel fitted into a Buchner flask. The resin was eluted with several aliquots of boiling distilled water. The eluate was cooled in a refrigerator and extracted with ether as described above. Although all of the α-DNP-amino acids

were removed from the resin by washing with boiling water, water-soluble derivatives and amino acids were still bound to the resin. In order to study this fraction, the resin was poured into a glass column (i.d. 14 mm.) and eluted with 0.8 ammonia. The eluate, and ether extract washings were taken to dryness in a rotary film evaporator at 40°C and after solution in acetone examined by T.L.C. In order to confirm that hydrolysis of the dinitrophenylated proteoglycan was complete, a sample of the combined water extracts and ammonia eluate was rehydrolysed with 5.7N-hydrochloric acid and the hydrolysate re-examined by thin layer chromatography. The combined ether extracts, after washing with 100 ml. of water, were taken to dryness and dissolved in 20 ml. of chloroform/acetic acid (99/1), and the absorption at 360 mμ in 1 cm. silica cells measured. This was used to calculate the number of N-terminal residues per gram of PP-L. This extract was also studied by quantitative T.L.C. All manipulations on DNP-derivatives were carried out in subdued lighting and where feasible the apparatus used was covered by a dark cloth. Corrections for the small destruction of α-DNP-amino acids which occurred during hydrolysis with the resin were applied after the method of Steven (1962).

III . RESULTS AND CONCLUSIONS

This final section comprises the analytical data on the various bovine cartilages and a brief discussion on their significance. The main part, however, will be devoted to the results of studies on the proteoglycan PP-L.

Cartilage Analyses

The results of these analyses are shown in Table XXIX.

Table XXIX

Type of Cartilage	Nasal	Arti-cular	Auri-cular	Epi-physeal	Meniscal
% moisture	74.0	78.7	70.5	76.0	65.5
% ash	13.1	7.7	5.4	35.0	2.5
% collagen	34.5	72.4	53.0	51.0	81.5
% elastin	0.0	0.0	18.8	0.0	0.0
% uronic acid	15.4	5.0	4.3	8.05	0.67
% chondroitin sulphate	43.0	14.0	12.0	22.4	1.87
% hexosamine	14.8	4.7	4.34	9.05	1.17
Collagen/chondroitin sulphate Ratio	0.81	5.2	4.4	2.3	43.5

i. All values are calculated as gm/100 gm. of moisture-and ash-free cartilage.

ii. Ash content is calculated as gm./100 gm. of moisture-free cartilage.

iii. Chondroitin sulphate calculated from the uronic acid content using the factor 2.8.

iv. Collagen is calculated from the amount of hydroxy-proline 'solubilised' by autoclaving, using the factor 7.46.

v. Elastin content is calculated from the hydroxy-proline 'resistant' to autoclaving, using the factor 52.3.

Several features are worthy of comment. Nasal, auricular and epiphyseal cartilages are classified by histologists as hyaline cartilages as they are considered to be non-fibrous and to consist largely of mucopolysaccharides. This is because the collagen fibres in hyaline cartilage do not stain with the usual light or electron microscope stains (Schubert and Hamerman, 1965). It is apparent from the analyses that this is far from true, particularly with respect to articular cartilage. It is interesting to note that auricular cartilage described as yellow elastic cartilage

contains 53% collagen but only 19% elastin. It would again appear that the morphological description is not borne out by the analyses.

The ash content appears to parallel the chondroitin sulphate content in all tissues apart from epiphyseal cartilage. The high figure for this tissue is similar to that quoted by Follis (1959), who found an ash content of 25% in rat epiphyseal cartilage. The value obtained will depend to a great extent on the age of the animal and the ease with which the calcified cartilage can be separated.

The hexosamine and uronic acid contents of all the cartilages studied, apart from meniscal cartilage, are very similar and this is in keeping with the finding that chondroitin sulphate is the main mucopolysaccharide in these tissues (Barker, Guy and Cruickshank, 1965). In meniscal cartilage the relatively high hexosamine content is a reflection of the high keratan sulphate in fibro-cartilage (Buddecke and Sziegoleit, 1964).

Apart from fibro-cartilage the collagen/chondroitin sulphate ratio is of the same order for all tissues. This similarity is interesting as these cartilages are subjected to very different types of stress and strain,

their functions are different and their mode of nourishment varied. This uniformity is possibly a reflection of the binding of chondroitin sulphate to collagen and would indicate that in auricular and epiphyseal cartilage as in articular cartilage, most of the proteinpolysaccharide is bound to collagen (Serafini-Fracassini and Smith, 1966).

More detailed analyses of these different types of cartilage for their content of the different mucopolysaccharides and of their proteinpolysaccharide complexes would yield valuable information on the chemical morphology of these tissues.

Proteoglycan Studies

Although early work on the proteoglycan PP-L suggested that it was probably homogeneous, this view has recently been challenged (Mashburn, Hoffman, Anderson and Meyer, 1965). The results of experiments on crude and purified forms of the proteoglycan will be given and these will be discussed with respect to the heterogeneity of PP-L.

(i) Purification with Cetyl Pyridinium Chloride

Purification of the crude proteoglycan (PP-L) by gradient elution of the precipitated PP-L - CPC complex

from a column of cellulose powder was carried out by the technique previously discussed. Fig. XXX is a typical elution chromatogram obtained. Apart from small peaks of material obtained during the CPC equilibration (? keratan sulphate) and during the first part of the gradient elution (? free protein) the complex was eluted as a single peak which was symmetrical apart from some tailing. A sample of PP-L prepared from bovine articular cartilage gave a similar chromatogram except that the protein peak was somewhat larger. A sample of PP-L purified by precipitation as its bismuth complex again gave a similar elution chromatogram except that neither the keratan sulphate nor protein peaks were present.

Samples of the CPC purified complex (PP-L - CPC) were assayed for their moisture, ash, nitrogen, protein, hexosamine and amino-acid composition by methods previously described. Samples were also examined by I.R. spectroscopy, electrophoresis, ultracentrifugation and for proteolytic activity. These results will be considered together with those obtained on the bismuth purified PP-L.

(ii) Use of bismuth nitrate as an E.M. stain.

The method of staining the proteoglycan with bismuth nitrate both in aqueous solution and in acetone have

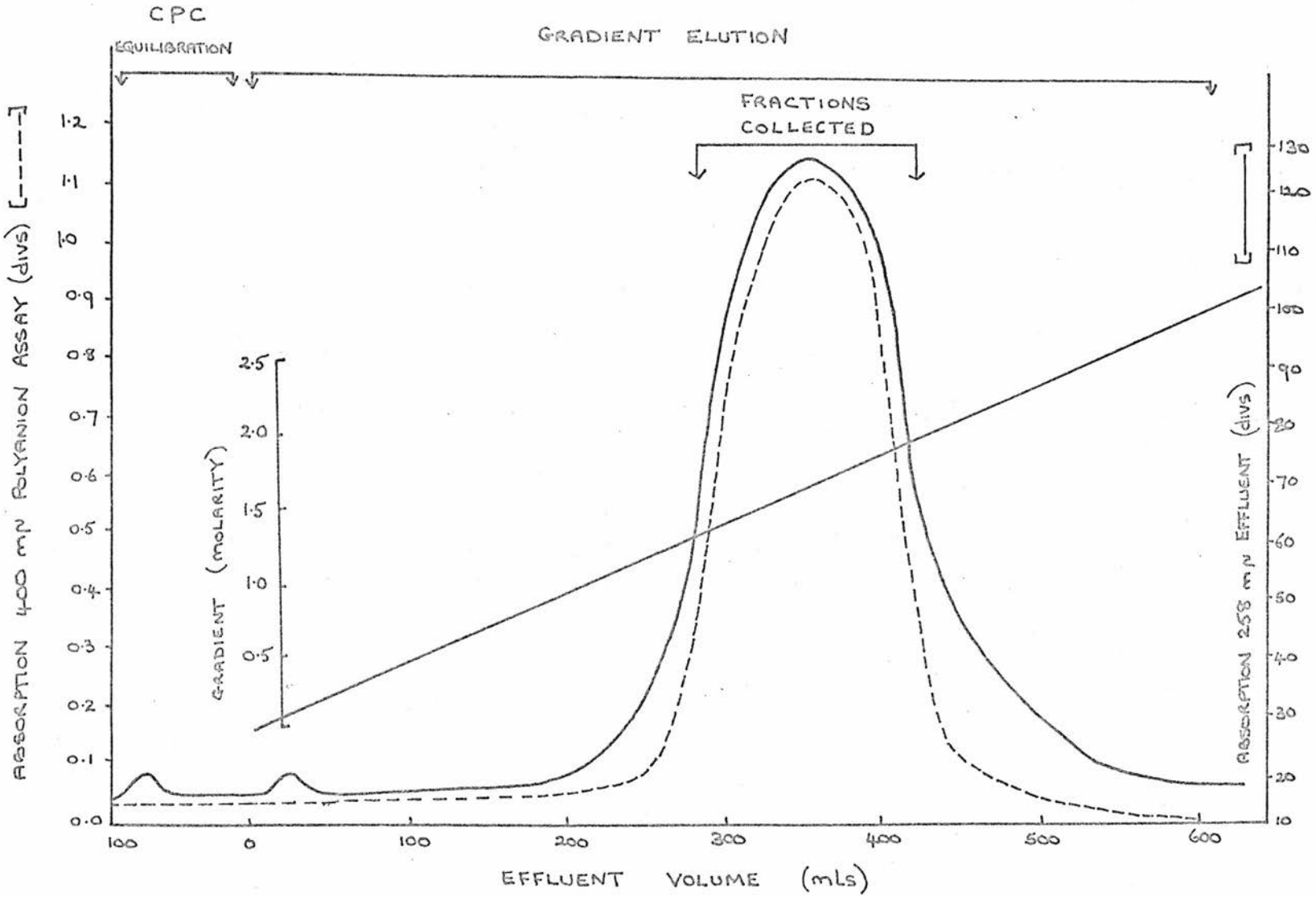


FIG XXX

ELUTION CHROMATOGRAM FOR PREPARATION OF PP-L-CPC

previously been described. It is believed that the bismuth ion combines with only the sulphate group of the proteoglycan in the same way that Albersheim and Killias (1963) used it to stain the phosphate moiety of nucleoproteins. Fig. XXXI is an E.M. photograph of a sample of PP-L stained with aqueous bismuth nitrate. This is interpreted as consisting of groups of closely packed dark spheroidal particles each of which consists of one or more chondroitin sulphate chains in coiled configuration. The average size of the particles is 47Å, 50% being between 45 and 50Å. The larger particles probably represent adjacent chondroitin sulphate chains which have become cross-linked in their coiled state.

Fig. XXXII is an E.M. photograph of a sample of PP-I stained with bismuth nitrate in acetone which, it is believed, shows the individual macromolecules. The majority of the stained elements appear as rows of single dark particles. The rows vary in length from 1100Å to 1500Å and each contains twenty to twenty-five particles. The particles vary in diameter from 15 to 47Å (average 30Å) whereas the unstained intervals between the particles vary in length from 17 to 60Å (average 36Å). There appears to be no correlation between the interval length and the size of the adjacent particles. It is considered

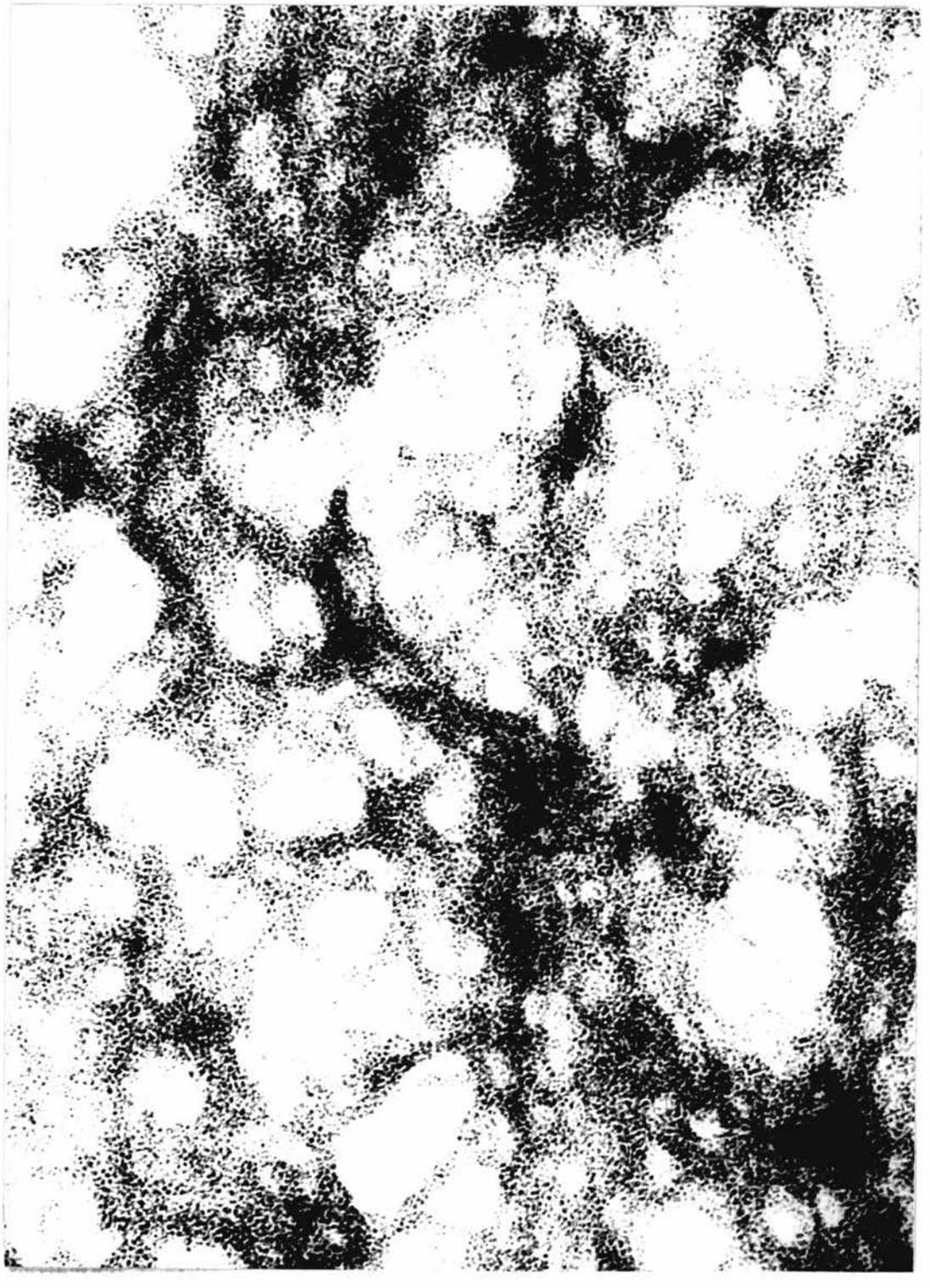


FIG XXXI

ELECTRON MICROGRAPH OF PP-L STAINED WITH AQUEOUS BISMUTH NITRATE [× 146,000]

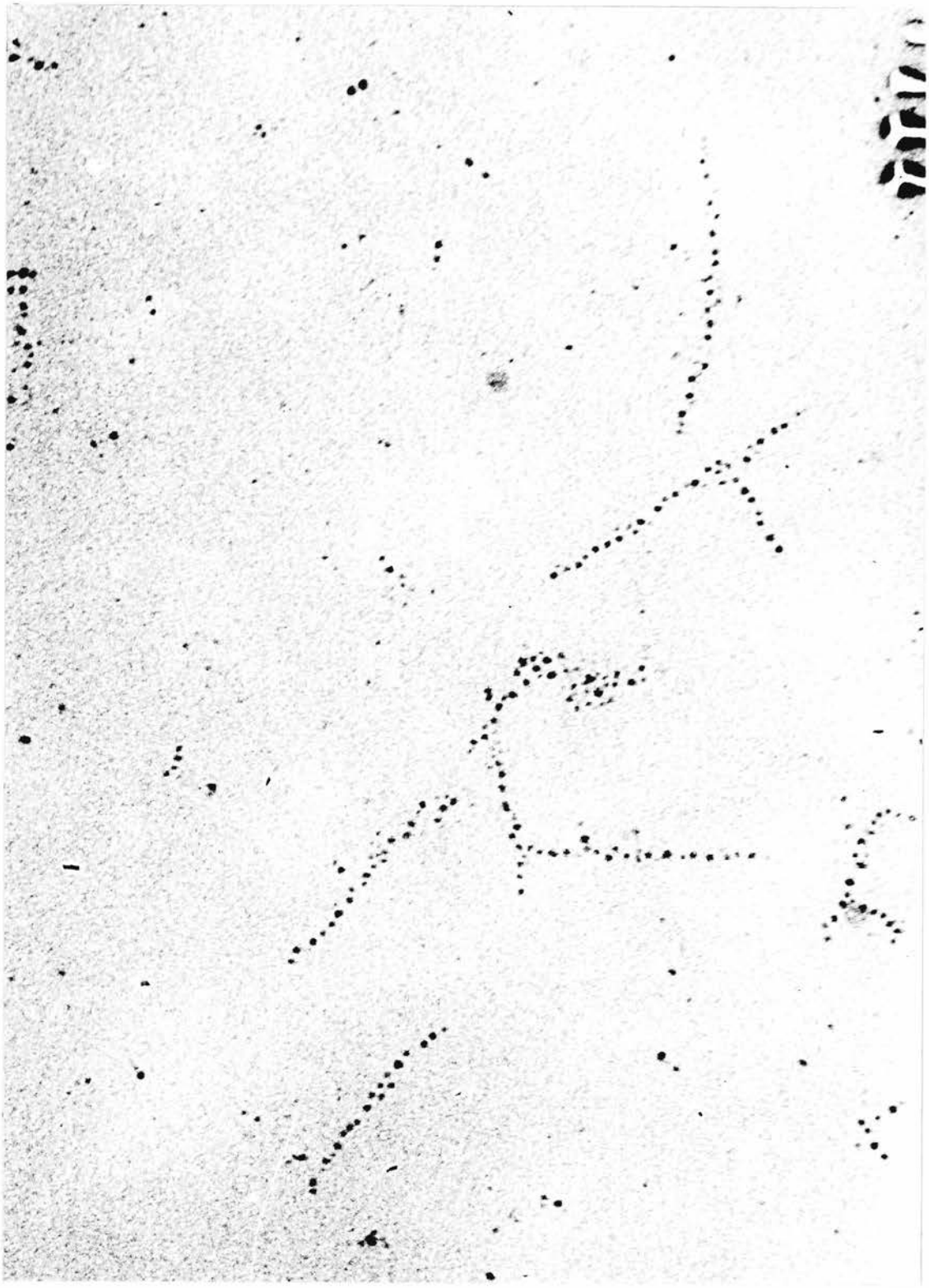


FIG XXXII

ELECTRON MICROGRAPH OF PP-L STAINED WITH BISMUTH NITRATE IN ACETONE [X 300,000]

that the rows of particles represent the proteoglycan macromolecules in which the protein core is unstained and the particles themselves are stained chondroitin sulphate chains in coiled configuration. These findings agree very closely with the model proposed by Partridge, Davis and Adair (1961) from physico-chemical studies on a product analogous to PP-L.

The use of bismuth nitrate in acetone appeared to give separate and discrete macromolecules, and thus this reagent was used as the basis of a purification procedure for PP-L. The details of the technique have been given in the previous section. The purified complex was analysed for its protein, nitrogen, hexosamine, moisture, ash and amino acid content. Samples of the bismuth purified complex (PP-L - Bi) were also examined by I.R. spectroscopy, electrophoresis, ultracentrifugation and for proteolytic activity.

(iii) The composition of crude and purified PP-L.

Table XXXIII gives the protein, nitrogen and hexosamine contents of the crude proteoglycan and samples purified with CPC, and with bismuth nitrate. It also indicates that proteolytic activity was found in the crude material but was not demonstrable in the

purified preparations. The sedimentation coefficients ($S^{\circ}_{20,w}$) of the crude and purified proteoglycans are also shown.

Table XXXIII

	PP-L - C	PP-L - CPC	PP-L - Bi
Protein ' content	17 - 20	16.0	14.1
Nitrogen '	5.8	5.8	5.6
Hexosamine '	29.3	-	30.5
Proteolytic activity	Present	Absent	Absent
Sedimentation coefficient	7.5 s	12.41 s	12.34 s

' Values are calculated as percentages of dry ash-free samples.

In order to determine the protein content of the various PP-I preparations and individual amino acid recoveries after acid hydrolysis, one sample of PP-L - C (sample 1) and one of PP-L - Bi (sample 5) were analysed after hydrolysis for 24, 36 and 72 hours at 110°C. The composition of the three PP-L - Bi (sample 5) hydrolysates are reported in Table XXXIV. A corrected protein content

of 14.17% was derived by extrapolation to zero hydrolysis time, of the summated amino acid contents, expressed as μg. of anhydro-amino acids per 10 mg. of dry ash-free material. The sample calculation procedure was applied to PP-L - C (sample 1) and since a very good agreement was obtained between the extrapolation slopes of the two preparations a correction coefficient of 1.108 was used for all the samples examined, in the calculation of their protein content from the amino acid composition of the 24 hour hydrolysates.

The amino acid composition values of the three PP-L - Bi (sample 5) hydrolysates converted to gm. of anhydro-amino acids per 100 gm. of the protein component are reported in Table XXXV. These values were used to correct the individual amino acid contents and to estimate the recoveries after 24 hours hydrolysis. Again good agreement was found with the recoveries calculated from the analytical data of PP-L - C (sample 1).

The extrapolated protein contents and the corrected amino acid compositions of the samples which have been analysed are reported in Table XXXVI.

Traces of tryptophan were found after alkaline hydrolysis only in the crude proteoglycan (PP-L-C). The absence of cysteine was confirmed by the failure to

Table XXXIV

Amino Acid Analysis of PP-L-Bi (Sample 5).

Amino Acid	Time of Hydrolysis 24 hr.	36 hr.	72 hr.
Hydroxyproline	0.00	0.00	0.00
Aspartic Acid	121.59	116.50	99.79
Threonine	74.81	71.47	55.70
Serine	87.32	73.00	69.28
Glutamic Acid	190.19	201.38	166.57
Proline	116.95	107.22	88.22
Glycine	77.88	78.38	57.87
Alanine	61.16	61.32	46.21
Valine	83.87	84.26	80.91
Cystine/2	0.00	0.00	0.00
Methionine	0.00	0.00	0.00
Isoleucine	63.80	60.59	59.83
Leucine	129.69	126.40	101.07
Tyrosine	47.55	33.09	15.06
Phenylalanine	63.45	70.85	54.17
Hydroxylysine	0.00	0.00	0.00
Lysine	57.48	57.09	43.46
Histidine	31.36	31.17	24.55
Arginine	71.96	70.38	67.33
Tryptophan	0.00	0.00	0.00
TOTALS	1,279.06	1,243.10	1,033.44

Protein content extrapolated to zero time: 14.17%

Composition expressed as µg. of anhydro-amino acid/10 mg. of PP-L-Bi (ash and moisture free)

Table XXXV

Amino Acid Composition of PP-L-Bi (Sample 5)

Amino Acid	Time of Hydrolysis			corrected values	recovery after 24hr. hydrolysis.
	24 hr.	36 hr.	72 hr.		
Hydroxyproline	0.00	0.00	0.00		
Aspartic Acid	8.58	8.22	7.04	9.36[a]	91.7%
Threonine	5.30	5.04	3.93	6.08[a]	87.2%
Serine	6.16	5.15	4.89	7.88[a]	78.3%
Glutamic acid	13.42	14.21	11.75	14.21[b]	94.4%
Proline	8.25	7.57	6.23	9.16[a]	90.1%
Glycine	5.50	5.53	4.08	5.53[b]	99.3%
Alanine	4.32	4.33	3.26	4.33[b]	99.8%
Valine	5.92	5.95	5.71	5.95[b]	99.5%
Cystine/2	0.00	0.00	0.00	0.00	-
Methionine	0.00	0.00	0.00	0.00	-
Isoleucine	4.50	4.28	4.22	4.52[a]	99.5%
Leucine	9.15	8.92	7.13	9.27[a]	98.7%
Tyrosine	3.36	2.34	1.06	4.24[a]	79.3%
Phenylalanine	4.48	5.00	3.82	5.00[b]	89.4%
Hydroxylysine	0.00	0.00	0.00	0.00	
Lysine	4.06	4.03	3.07	4.15[a]	97.8%
Histidine	2.21	2.20	1.73	2.25[a]	98.2%
Arginine	5.08	4.97	4.75	5.18[a]	98.0%
Tryptophan	0.00	0.00	0.00	0.00	-
TOTALS	90.29	87.74	72.67	97.11	

Composition expressed as gm. of anhydro-amino acid/100 gm. of the protein component.

a = Extrapolation to zero time.

b = Greatest value.

Table XXXVI

Amino Acid Analysis of PP-L-C, PP-L-CPC and PP-L-Bi.

	PP-L-C			PP-L-CPC	PP-L-Bi	
SAMPLE:	1	2	3	4	5	1
Hydroxyproline '	0.00	0.00	0.00	0.00	0.00	0.00
Aspartic Acid '	9.98	10.34	10.19	10.01	9.36	9.71
Threonine '	5.46	5.45	5.58	5.56	6.08	5.55
Serine '	7.11	7.31	6.81	7.46	7.88	7.87
Glutamic Acid '	14.59	15.21	15.23	15.93	14.21	14.29
Proline '	9.32	7.82	9.11	9.14	9.16	9.23
Glycine '	6.90	5.51	5.82	5.61	5.53	6.10
Alanine '	5.00	5.37	4.64	4.99	4.33	4.36
Valine '	5.93	6.88	6.06	5.74	5.95	5.90
Cystine/2 '	1.22	TR	TR	0.00	0.00	0.00
Methionine '	1.54	0.00	0.00	0.00	0.00	0.00
Isoleucine '	3.95	4.87	4.92	4.50	4.52	4.80
Leucine '	8.35	9.54	9.98	9.05	9.27	9.43
Tyrosine '	4.16	4.38	4.70	4.20	4.24	3.67
Phenylalanine '	4.43	4.94	6.36	5.61	5.00	4.98
Hydroxylysine '	0.00	0.00	0.00	0.00	0.00	0.00
Lysine '	4.02	4.94	5.43	4.31	4.15	3.82
Histidine '	2.19	2.11	2.26	2.22	2.25	2.22
Arginine '	7.12	5.59	6.86	6.21	5.18	4.93
Tryptophan '	TR	-	-	0.00	0.00	0.00
Protein *	20.12	18.18	17.48	15.96	14.17	14.14

' Values expressed as gm. of anhydro-amino acid/100 gm. of protein.

* Values calculated as percentages of dry ash-free samples.

demonstrate the presence of thiol groups in any of the samples (less than 10^{-10} moles of -SH groups/mg. of proteoglycan) using the technique of Ellman (1959).

Table XXXVII gives the amino acid composition, expressed as moles/1000 moles for PP-L - Bi (mean of samples 1 and 5). The figures obtained by other workers for the composition of the proteoglycan are given for comparison. Muir (1958) used a highly purified proteoglycan isolated from pig tracheal cartilage. Scheinthal and Schubert (1963) report analyses on the crude PP-L complex isolated from bovine nasal cartilage; Partrdige, Whiting and Davis (1965) on a preparation of PP-L purified by DEAE chromatography; and Buddecke, Kröz and Lanka (1963) report the analysis of a sample of bovine nasal cartilage PP-L, purified by a CPC chromatographic technique similar to that reported in this work.

Amino acid analyses for bovine and rat costal cartilage proteinpolysaccharide have been recorded by Campo and Dziewiatrowski (1962) and by Gross, Matthews and Dorfman (1960) respectively. The compositions are very similar to those recorded in Table XXXVII. It appears that the protein moiety of the protein-chondroitin 4-sulphate complex has a similar

Table XXXVII

Amino Acid Composition of PP-L-Bi compared with reported values

Amino Acid	A	B	C	D	E
Hydroxyproline	0.00	0.00	0.00	0.00	0.00
Aspartic Acid	89.96	92.1	94.6	116.8	80.08
Threonine	62.45	55.3	53.2	54.1	52.39
Serine	98.19	105.5	90.5	97.8	84.11
Glutamic Acid	119.83	125.0	122.3	123.0	139.49
Proline	102.80	-	93.0	62.1	102.96
Glycine	110.63	116.7	123.5	100.1	119.47
Alanine	66.37	93.0	69.4	72.1	71.50
Valine	64.89	73.3	57.5	63.0	67.73
Cystine/2	0.00	Trace	14.4	13.1	0.00
Methionine	0.00	-	9.6	-	2.44
Isoleucine	44.70	49.4	35.7	36.8	38.65
Leucine	89.71	103.7	73.7	76.5	79.27
Tyrosine	26.32	20.2	25.8	30.6	38.74
Phenylalanine	36.81	54.6	36.5	33.9	42.48
Hydroxylysine	0.00	-	-	-	-
Lysine	33.76	27.0	32.5	34.4	22.23
Histidine	17.69	15.7	20.4	20.9	5.62
Arginine	35.14	40.2	47.3	46.5	45.50
Tryptophan	0.00	-	-	-	-
Protein	14.1%	10.0%	15.5%	7.7%	17.0%

A = This work

B = Muir (1958)

C = Scheinthal and Schubert (1963)

D = Partridge, Whiting and Davis (1965)

E = Buddecke, Kröz and Lanka (1963).

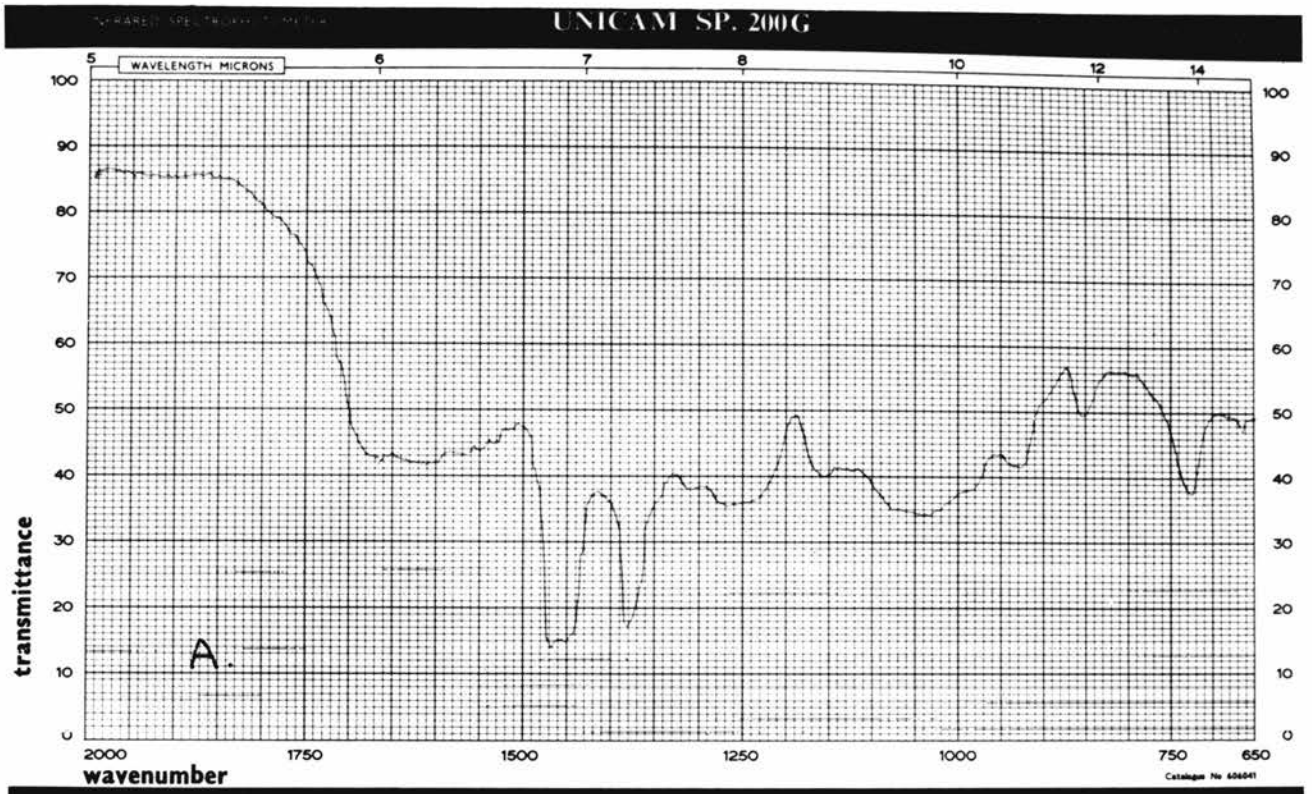
composition in whatever tissue it is present, in the rat, ox and pig.

In spite of the markedly differing protein contents, the amino acid compositions obtained by the different workers using dissimilar purification techniques are very similar.

(iv) I.R. studies on crude and purified PP-L.

Fig. XXXVIII a and b are the I.R. spectra of samples of PP-L prepared from bovine articular and nasal cartilage respectively. Fig. XXXIX a and b are the I.R. spectra of samples of PP-L - Bi and PP-L - CPC respectively. These absorption spectra are very similar and are attributable to the chondroitin 4-sulphate content of the complexes. They are identical to the I.R. spectrum of a sample of chondroitin 4-sulphate isolated from PP-L. The spectra are identical to those reported by other workers for the crude proteoglycans (Gross, Matthews and Dorfman, 1960; Campo and Dziewiatkowski, 1962; Scheinthal and Schubert, 1963). The significant peaks are 1648 and 1410 cm.$^{-1}$ (carboxylate), 1230 to 1260 cm.$^{-1}$ (sulphate) and 928, 852 and 725 cm.$^{-1}$ (axial 4-sulphate). There are no absorption peaks characteristic of equatorial 6-sulphate (Hirano, Hoffman and Meyer, 1961; Turvey, 1965; Orr, 1954; Neeley, 1957).

FIG XXXVIII INFRA-RED SPECTRA OF PP-L PREPARED FROM BOVINE NASAL CARTILAGE (A.) AND FROM BOVINE ARTICULAR CARTILAGE (B.)



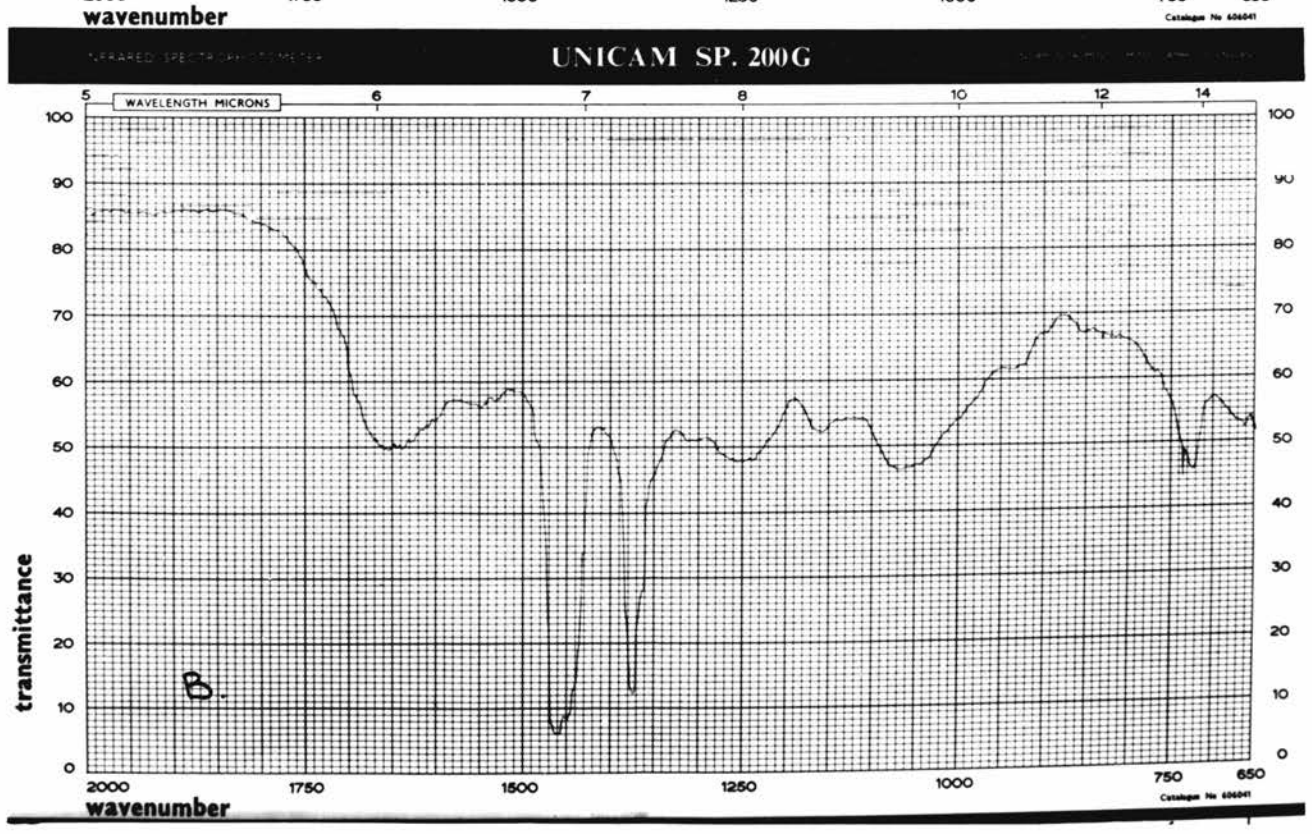
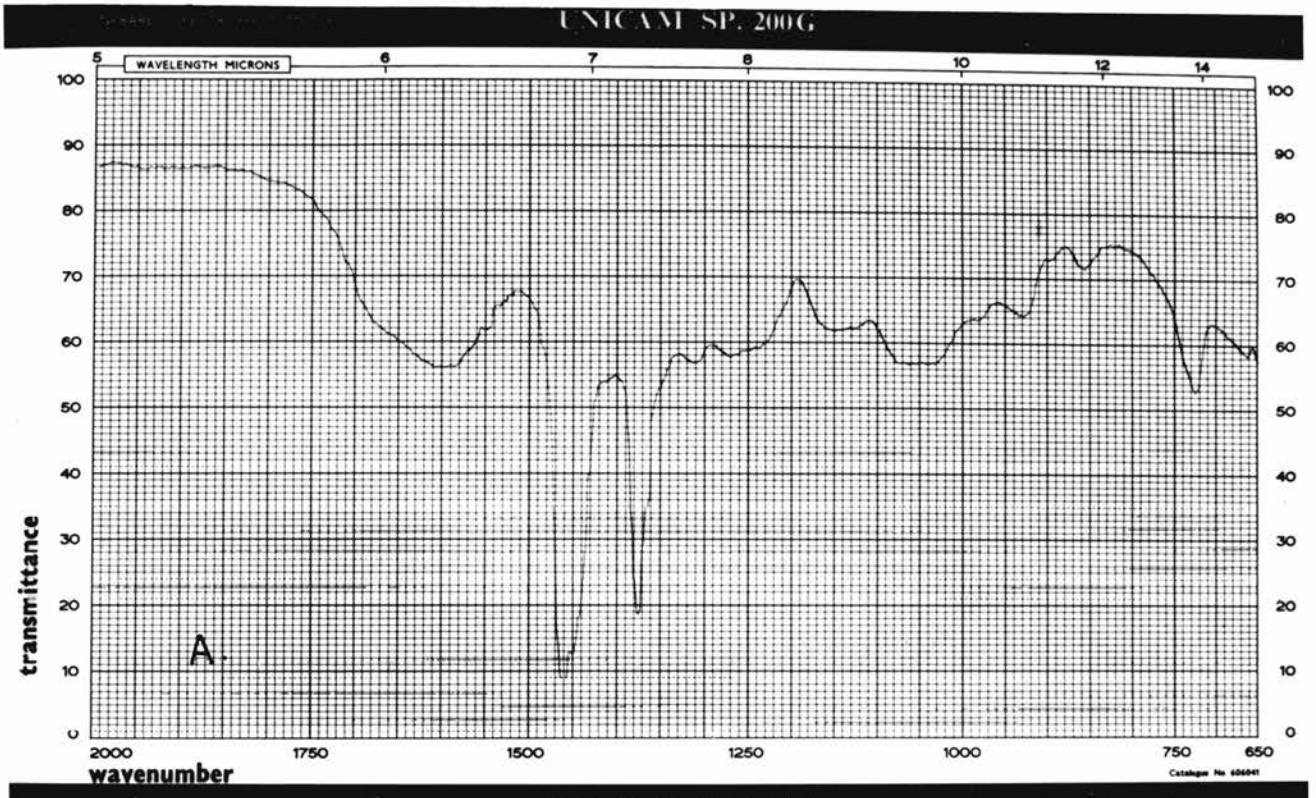
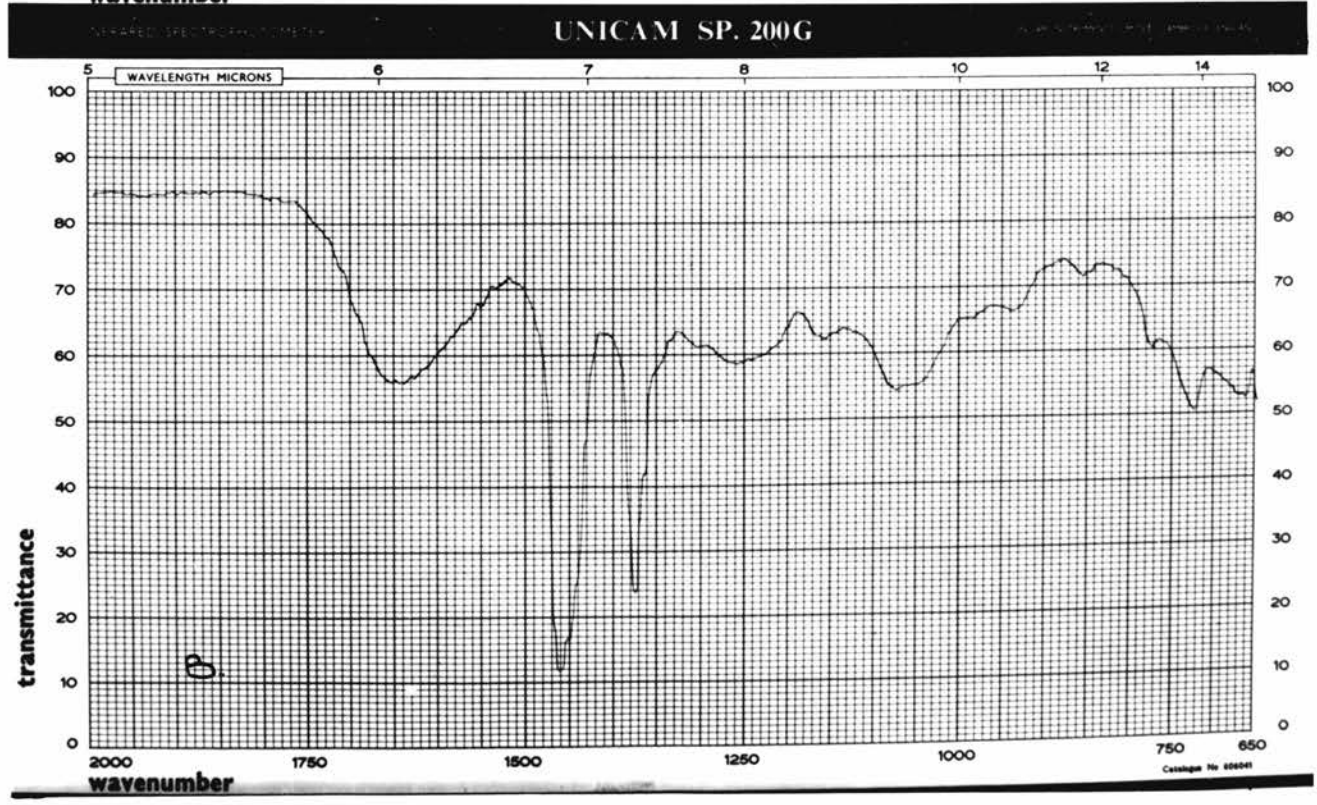


FIG XXXIX INFRA-RED SPECTRA OF BISMUTH PURIFIED PP-L (A.) AND CPC PURIFIED PP-L (B.)





(v) Electrophoretic Studies

Using cellulose acetate paper as the supporting medium, samples of the crude and purified complex were subjected to electrophoresis under the conditions previously described. Fig. XL shows photographs of the electrophoretic strips of samples of PP-L-C, PP-L-CPC and PP-L-Bi which have been run at the same time. All appear to migrate as a single peak at a similar rate.

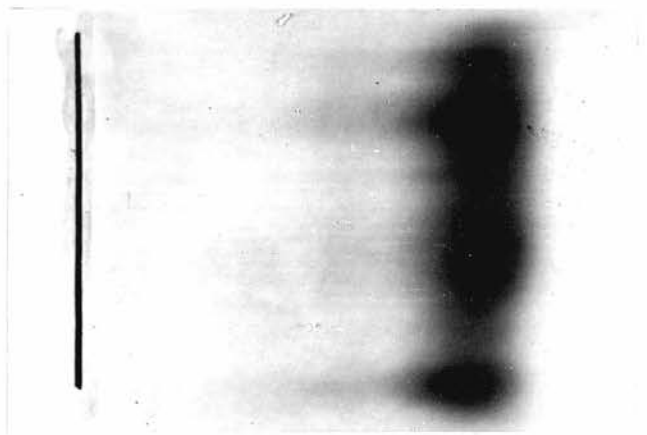
(vi) Sedimentation coefficients of PP-L-CPC and PP-L-Bi

The sedimentation coefficients at infinite dilution ($S^{o}_{20,w}$) of PP-L-CPC and PP-L-Bi obtained by linear extrapolation of 1/S or $S(\eta/\eta_o)$ against c to c=0 by the method of least squares were 12.41s. and 12.34s respectively. The determination of $\bar{V}$ gave an average value of 0.686 ml./gm. for both preparations.

(vii) Analysis of the acetone supernatant

Chromatographic analyses carried out on the organic material liberated from PP-L-C by the bismuth nitrate in acetone and isolated from the acetone supernatant, showed the presence of several peptides, a large hexosamine spot (Morgan-Elson reaction) and significant amounts of the following free amino acids: isoleucine, phenylalanine,

a. BISMUTH PURIFIED PP-L



b. CPC PURIFIED PP-L

c. CRUDE PP-L

FIG XL

PHOTOGRAPHS OF ELECTROPHORETIC STRIPS OF CRUDE AND PURIFIED PP-L

tyrosine, arginine, valine, alanine, glycine, glutamic acid, leucine and aspartic acid.

(viii) Amino-group analysis of PP-L-C and PP-L-Bi

From the amount of sodium hydroxide used up during the titration of the proteoglycans with FDNB, it is possible to calculate, very approximately, the number of reactive $-NH_2$ groups. Table XLI shows the number of titratable groups measured in both PP-L-C and PP-L-Bi at pH 6.9 and pH 8.4. These figures are in keeping with those of Cessi and Bernardi (1965) who found approximately 100 $-NH_2$ groups/molecule of proteinpoly-saccharide complex.

Table XLI

	pH 6.8	pH 8.4
PP-L-C	73	125
PP-L-Bi	42	85

Values expressed as reactive groups (moles,$-NH_2$) per 10^6 gm. (moisture and ash free) proteoglycan.

From the optical density of the total ether extracts, i.e. of the α-DNP amino acids, it is possible to calculate approximately the total number of N-terminal groups per 10^6 gm. of proteoglycan. However, this crude ether extract is contaminated by ether-soluble breakdown products

from the resin and to obtain a reliable figure it would be necessary to purify this extract. However, the figures expressed as moles of DNP-amino acids/10^6 gm. of proteoglycan are given in Table XLII.

Table XLII

	pH 6.8	pH 8.4
PP-L-C	2.8	4.74
PP-L-Bi	1.27	3.1

Values expressed as amino acid derivatives (moles) per 10^6 gm. (moisture and ash free) proteoglycan.

Chromatographic separation of the N-terminal amino acids enabled an accurate value for the amount of each N-terminal amino acid to be calculated for the bismuth nitrate purified proteoglycan. Table XLIII gives the results of these quantitative end-group analyses.

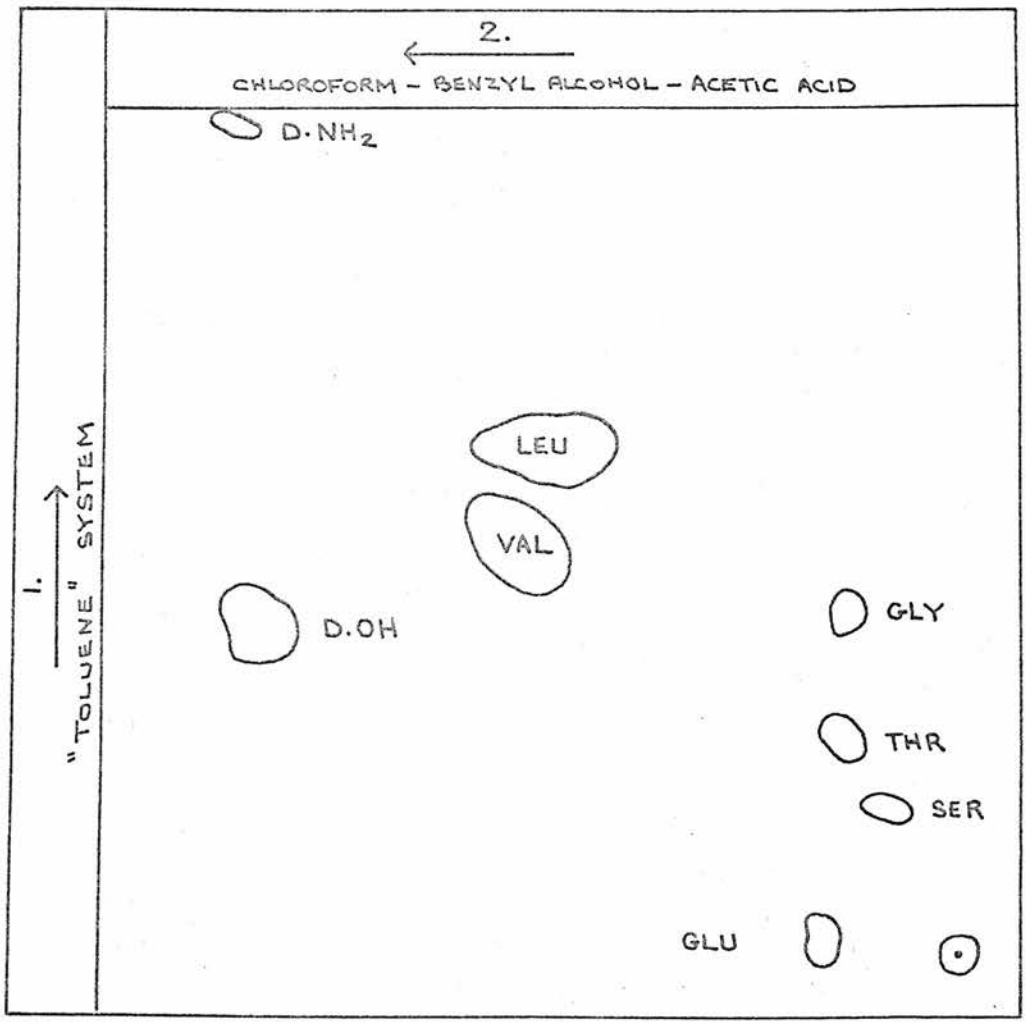
Table XLIII

Aspartic Acid	0.157
Threonine	0.112
Serine	0.183
Glycine	0.055
Valine	0.490
Leucine	0.592
TOTAL	1.589

Values expressed as moles of amino acid/10^6 gm. of (moisture and ash free) PP-L-Bi.

Fig. XLIV is a tracing of the thin-layer chromatogram obtained from such an experiment. When the N-terminal amino acids from the unpurified PP-L-C were chromatographed, the above amino acid derivatives, together with the following, were found to occupy the end-group position: glutamic acid, alanine, phenylalanine and isoleucine.

The amino acids and water-soluble DNP-amino acid derivatives released by the resin hydrolysis of the dinitrophenylated derivatives of both PP-L-C and PP-L-Bi were examined by thin-layer chromatography. As would be expected from the quantitative amino acid analyses no significant difference in composition between the two samples was found. Examination of the chromatograms by ultra-violet light before staining with ninhydrin, revealed only ϵ DNP-lysine. No O-DNP-tyrosine was visualised. When the plates were examined after ninhydrin development, they revealed no free lysine and clearly demonstrated free tyrosine. Further hydrolysis of resin hydrolysate, with 5.7N-hydrochloric acid to break down any DNP-peptides, confirmed the above findings.



THIN LAYER CHROMATOGRAM OF ∝ DNP AMINO ACIDS FROM SAMPLE OF BISMUTH PURIFIED PP-L

D·OH DINITROPHENYL

D·NH$_2$ DINITROANILINE

FIG XLIV

Samples of O-DNP-tyrosine subjected to resin hydrolysis by the same method behaved in the expected manner when subjected to T.L.C. and thus the failure of the hydroxyl group of tyrosine to react with FDNB is probably not an artefact (Steer, 1966 - personal communication). Similarly the failure to find free lysine in the chromatograms makes it very unlikely that the ϵ-amino group is involved in any linkage in the native molecule. It is proposed to discuss the possible significance of the failure to dinitrophenylate the hydroxyl group of tyrosine in the next section.

DISCUSSION

The protein content of PP-L decreases from an average value of 18.6% to 14.1% on purification of the proteoglycan by precipitation with bismuth nitrate in acetone. The alternative purification procedure, in which the PP-L is precipitated with CPC and selectively resolubilised, gives a material with an intermediate protein content of 16.0%. However, as shown in Table XXXVI, the amino acid composition of the protein moiety changes very little on purification. A decrease in the arginine content and the absence of tryptophan, methionine and cystine are the main differences between the crude and purified complexes. Some of the slight differences in amino acid composition which are evident in different samples and preparations, can be attributed to the contamination of PP-L-C by free amino acids and peptides and by trace amounts of at least two proteolytic enzymes. Steven and Tristram (1962) showed that peptides and several free amino acids can be released from highly purified preparations of bovine collagen by precipitation with acetone at an acid pH. The presence of these compounds might therefore be considered to be a normal physiological

constituent of connective tissues. The similarity in amino acid composition of crude and purified PP-L preparations, in spite of the decrease in protein content with purification, suggests that a proportion of the proteoglycan macromolecules in PP-L-C may have a reduced chondroitin sulphate content. These macromolecules may be naturally occurring degradation products in which some of the chondroitin sulphate chains have been cleaved from the protein core and then excluded from the domain of the macromolecule. The enzyme isolated by Tourtellotte, Campo and Dziewiatkowski, (1963), from various cartilaginous tissues, which has been shown to cleave the chondroitin 4-sulphate from the protein core may be implicated in this role.

Examination of the amino acid composition of PP-L-Bi expressed as residues per 1000 residues (Column A, Table XXXVII) reveals the existence of several groups in which the amino acids are present in approximately equimolar concentrations. Serine, glutamic acid, proline and glycine, each appears to occur with the frequence of one in ten residues; aspartic acid and leucine as one in eleven residues; threonine, alanine and valine as one in sixteen residues and, finally,

phenylalanine, lysine and arginine as one in twenty-nine residues. It is interesting to note that equimolarity of serine, glutamic acid, proline and glycine was reported by Anderson, Hoffman and Meyer (1965) in chondroitin 4-sulphate-peptide complexes which had been produced from a preparation of bovine nasal chondromucoprotein by exhaustive proteolysis. They also noted a similar pattern in the chondroitin 6-sulphate-peptide complexes, similarly isolated from shark cartilage. When these derivatives were dinitrophenylated, several amino acids were present as end-groups indicating heterogeneity of these small peptides.

These data suggest that the protein core of PP-L may be made up from a limited number of peptide 'sub-units' of slightly differing amino acid composition and sequence, but in which the same amino acids are involved in the polysaccharide linkage region. The amino acid end-group analysis performed on PP-L-Bi (Table XLIII) shows that six amino acids can be consistently detected as α-DNP derivatives. These findings might suggest that PP-L is a mixture of proteoglycan macromolecules differing in their protein

cores. However, this view is not consistent with the striking similarity in amino acid composition of the crude and purified chondromucoprotein preparations. An alternative interpretation could be that the protein core of PP-L is made up of peptide, 'sub-units', the sequential arrangement of which in the macromolecule is not strictly determined. If this were so, each 'sub-unit' would occupy the external position in the macromolecule, at the free amino-end, with a frequency proportional to its occurrence in the whole protein core. If this second interpretation is correct, the number average molecular weight of the macromolecule can be calculated from the total number of moles of $-NH_2$ terminal amino acids per 10^6 gm. of PP-L. The value of 6.3×10^5 so obtained, is in good agreement with that (5.5×10^5) calculated by Buddecke, Krötz and Lanka (1963) from sedimentation data for a product analogous to PP-L-CPC. The similarity between PP-L-Bi and PP-L-CPC is emphasised by the identical values for their $S^{o}_{20,w}$ and the results of I.R. and electrophoretic studies.

Since the protein content of PP-L-Bi is 14.1%, the molecular weight of the protein core can be

calculated as 8.9×10^4, leaving a total molecular weight of 5.4×10^5 for the carbohydrate moiety. The chain weight of the chondroitin sulphate chains, isolated by papain digestion has been reported as ranging between 2.2×10^4 and 2.8×10^4 (Matthews, 1956; Partridge, Davis and Adair, 1961; Buddecke, Krötz and Lanka, 1963). Therefore, some 20 to 24 chondroitin sulphate chains should be present in the proteoglycan macromolecule. This number is in remarkable agreement with the results of electron microscope examination of PP-L-Bi which showed 20 to 25 particles, interpreted as being chondroitin sulphate chains, arranged in single rows 1100 to 1500Å long.

Schubert visualises the protein core as a comb-like structure. At approximately 50 residue intervals is a side chain of 20 amino acid residues, to the end of which is attached the chondroitin 4-sulphate molecule. He suggests that this side chain is split from the central protein core by alkalis. In view of the fact that the hydroxyl group of tyrosine is 'non-reactive', with respect to FDNB, it is possible that the tyrosine residue could occur in each pepetide 'sub-unit' as a normal sequence, the hydroxyl group

forming an alkali-labile link with the carboxyl group of the C terminal amino acid in the 20 residue side chain. It is interesting to note that one can calculate from the amino acid analysis that there would be approximately 25 tyrosine residues in a protein core of weight 8.9×10^4, i.e. the number of chondroitin 4-sulphate chains visualised by electron microscopy and calculated from the molecular weight of the whole complex. There is, however, no precedent for such a linkage in Molecular Biology, but it would be readily susceptible to experimental verification.

IV. SUMMARY

1. Samples of epiphyseal, articular, auricular, nasal and meniscal cartilage from a six-month steer were assayed for their moisture, ash, hexosamine, uronic acid, collagen and elastin contents and the results discussed in the light of their classical anatomical structure.

2. Chondromucoprotein from bovine nasal cartilage was purified by cetyl-pyridinium chloride and by bismuth nitrate in acetone. This latter reagent was explored as an electron microscope stain for the chondromucoprotein.

3. The chondromucoprotein was visualised as an unstained protein core, 1100 - 1500Å in length, along which were 20-25 chondroitin sulphate chains in coiled configuration which appeared as dark particles.

4. Amino acid composition of crude and purified preparations were compared and little difference was found, in spite of the decrease in protein content on purification.

5. Amino acid analysis of the bismuth-purified material revealed the existence of four groups of amino acids in which these were present in approximately equimolar concentration.

6. Studies on the dinitrophenylated derivative of the same material showed six amino acids in the N-terminal position and indicated that the hydroxyl group of tyrosine was probably substituted.

7. A molecular weight of 6.3×10^5 for the protein-polysaccharide complex was calculated from the quantitative end-group analysis.

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