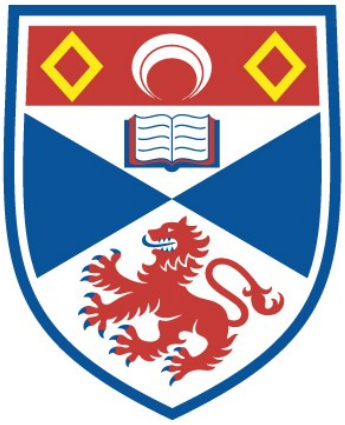
THE CHARACTERISATION OF PROTROHEPARIN IN BOVINE LIVER CAPSULE

Christopher John Branford White

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1973

THE CHARACTERISATION OF PROTEOHEPARIN IN BOVINE LIVER CAPSULE

A Thesis

presented by

CHRISTOPHER JOHN BRANFORD WHITE

to

the University of St. Andrews in application for the Degree of Doctor of Philosophy

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. A. Serafini-Fracassini.

CERTIFICATE

I hereby certify that Christopher John Branford White has spent nine terms engaged in research work under my direction and that he has fulfilled the conditions of Ordinance No.16 (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I first matriculated at the University of St. Andrews in October 1968, and graduated with the degree of Bachelor of Science, First Class Honours in Biochemistry in June 1970. I matriculated as a research student in the Department of Biochemistry, University of St. Andrews, in October 1970.

ACKNOWLEDGMENTS

I should like to express my sincere gratitude to Dr. A. Serafini-Fracassini for his continued encouragement and advice throughout the course of this work. My thanks are also due to: Mr. J. C. Hunter, Mr. C. Armit and Mr. W. Blyth for their able technical assistance, Dr. D. Thirkell for guidance in culturing F. heparinum, Mr. R. Henderson, Dundee Royal Infirmary, for biological activity determinations, Mr. B. O'Malley, St. Andrews Abattoir, for supplying bovine livers and to Mrs. M. McAlister for typing this thesis.

THE CHARACTERISATION OF PROTEOHEPARIN

IN BOVINE LIVER CAPSULE

C O N T E N T S

INTRODUCTION

INTRODUCTION

Heparin is a unique hetero-polysaccharide found in mammalian connective tissue within the granules of highly differentiated cells (mast cells) which are particularly abundant in serous membranes. The structure of this polysaccharide has been elucidated but uncertainties still exist regarding some chemical and physical chemical parameters.

In 1916 McLean first extracted from dogs' liver, a substance exhibiting anticoagulant activity which was later named 'Heparin' by Howell and Holt (1918-19). The extraction procedure about this time involved treatment of the tissue with either alkali or proteases [Charles & Scott, 1933]. Early chemical analyses revealed the presence in heparin of nitrogen, hexuronic acid and hexosamine [Jorpes & Bergström, 1936]. Moreover, a high percentage of bound sulphate was detected and a tetrasaccharide repeating unit was proposed which contained equimolar amounts of glucosamine and hexuronic acid with five sulphate groups [Wolfrom *et al.*, 1943]. Both glucuronic and

iduronic acid residues have been shown to be present in the structure, but their actual locations are still uncertain [Cifonelli & Dorfman, 1962; Radhakrishnamurthy & Berenson, 1963; Lindahl, 1966; Wolfrom et al., 1969; Perlin & Sanderson, 1970].

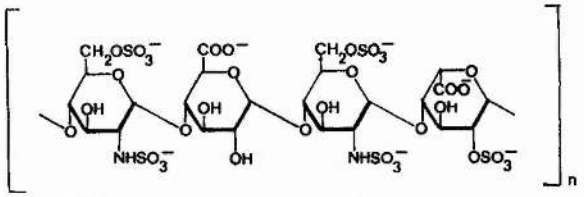
The most recent advances in structural analysis have resulted from the combination of chemical and enzymic techniques. In particular the degradation of heparin by heparinase has been found to be of great value in the breakdown of heparin chains into small oligosaccharides, which are more amenable to chemical analysis.

Crude heparinase was first isolated by Korn and Payza (1956a,b) from Flavobacterium heparinum induced on heparin. Fractionation of this enzyme preparation [Linker & Hovingh, 1965; Dietrich, 1968; Hovingh & Linker, 1970] yielded at least 4 or 5 enzymes which probably comprise the active complex. The initial degradative reaction involves an eliminase, which requires for its specificity the presence of O-sulphate and sulphamido groups. Other enzymes present act on

such degradation products with both sulphatase and glycuronidase activities [Dietrich, 1969; Hovingh & Linker, 1970].

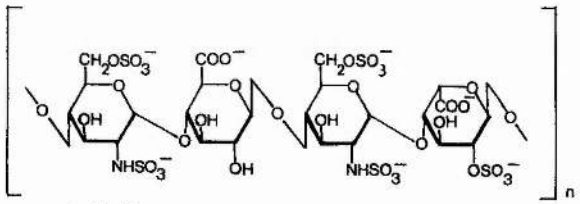
Chemical and physical analyses of enzymic degradation products [Dietrich, 1968; Linker & Hovingh, 1970] have clearly confirmed that the tetrasaccharide proposed by Wolfrom is the basic structural unit of heparin, although the degree of sulphation has been questioned [Linker & Hovingh, 1972]. In particular, some controversy still exists regarding the presence of the fifth sulphate group which has a reported location on the C-2 carbon of iduronic acid [Lindahl & Axelsson, 1971]. It is worth noting in this connection that titration studies [Herbert & Marini, 1963] have indicated the presence of unbound sulphate ions which may affect S : glucosamine molar ratio in most preparations.

The glucosamine and hexuronic acid units are joined by α-(1,4)-glycosidic linkages [Danishefsky & Steiner, 1965] and the structure of the tetrasaccharide unit which is at present generally accepted is illustrated in figure 1.



α–[1,4]–Glycosidically Linked Tetrasaccharide Repeating Unit.

However it is worth noting that Helting and Lindahl (1971) have reported that between one-third and all of the glucuronate residues of heparin are cleaved by β-glucuronidase when located at the reducing end of oligosaccharides. This indicates that a large portion of these residues are β-glycosidically linked and the structure of the repeating would therefore be:-



α,β–[1,4]–Glycosidically Linked Tetrasaccharide Repeating Unit

Figure 2

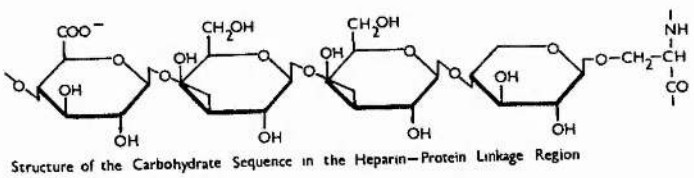
Recent evidence from nuclear magnetic resonance experiments seem to support the structure shown in figure 1, since only the presence of alternating α-(1,4)-glycosially linked biose units were observed [Perlin et al., 1972].

Although it is generally accepted that a major proportion of the heparin chains are made up of such tetrasaccharide repeating units, a number of micro-heterogeneities are evident. In particular, the degree of sulphation does not appear to be uniform along the structure, in that the portion of the chain in the vicinity of the potential reducing end contains fewer sulphated residues in comparison to other areas in the chain [Lindahl, 1966]. A similar pattern is observed in the distribution of iduronic acid residues along the polymer [Lindahl & Axelsson, 1971].

Results from such chemical findings have shed little light on the steric structure of the heparin molecule; however, on the strength of nuclear magnetic resonance, optical rotatory dispersion and electron microscopy, Hirano (1972) proposed that the heparin

chain was organised in a right-hand helical conformation. As yet this model has not been confirmed by X-ray analysis.

Commercially prepared heparin chains have been shown to be bound to a peptidyl backbone through their potentially reducing ends. In this region the presence of the alternating tetrasaccharides ceases and the glucuronyl residue next to the last repeating unit is glycosidically linked to the trisaccharide 3-O-β-D-galactosyl-4-O-β-D-galactosyl--D-xylose which in turn bridges the gap between the heparin chain proper and the peptide (figure 3). The terminal xylose is O-glycosidically linked to the hydroxyl group of a seryl residue which lies within the peptidyl backbone [Lindahl *et al.*, 1965; Lindahl & Rodén, 1965; Lindahl, 1966].



Structure of the Carbohydrate Sequence in the Heparin–Protein Linkage Region

Figure 3

This type of linkage is found to occur in all sulphated glycosaminoglycans with the exception of keratan sulphate and so may be regarded as a structural characteristic of these macromolecules [Muir, 1958; Gregory et al., 1964; Anderson et al., 1965; Lindahl & Rodén, 1966; Rodén & Armand, 1966; Rodén & Smith, 1966; Helting & Rodén, 1968]. At this point it may be worth outlining the main features of cartilage proteoglycan which are the best characterised compounds of this type in order to assess and compare any structural similarities with those of proteoheparin.

The proteoglycans of cartilage contain both chondroitin sulphate and skeletal keratan sulphate as macromolecular complexes in which several glycosaminoglycan chains are bound to a protein moiety [Schatton & Schubert, 1954]. Ideas regarding the molecular organisation and structural significance of these macromolecular complexes are at present somewhat speculative although over the years a number of interesting theories have been postulated.

Mathews and Lozaityte (1958) proposed a structural model in which the complex was visualised as a comb-like unit consisting of several glycosaminoglycan chains each bound through their potentially reducing ends to a protein backbone. Evidence from electron microscopy supporting this model has been demonstrated by Serafini-Fracassini and Smith (1966) and by Rosenberg et al., (1970a). However, recently it has been shown that chondroitin sulphate chains are arranged on a protein backbone in pairs, this arrangement is normally referred to as a 'doublet' [Anderson et al., 1965; Luscombe & Phelps, 1967; Hascall & Riolo, 1972]. In a chondroitin sulphate doublet the two glycosaminoglycan chains are separated by less than ten amino acid residues and each doublet is further separated from its neighbouring doublet by a longer section of peptide, estimated to consist of about thirty-five amino acid residues and this overall molecular weight of this complex is approximately 47,000 daltons [Mathews, 1971]. Chemical evidence suggests that the primary structure of this polypeptide core is similar in all species and thus it also appears that

the sequence of amino acids in the doublet is a requirement for the enzymic recognition of specific seryl residues during biosynthesis [Mathews, 1971]. In fact it has been demonstrated that the xylosyl transferase of chick cartilage 'recognises' specific residues on the core protein of bovine proteoglycan [Baker et al., 1971; Stoolmiller et al., 1972]. Amino acid sequences from proteochondroitin sulphate and proteodermatan sulphate show some form of similarity which suggests some degree of homogeneity within these macromolecular complexes.

The chemical composition of the complex varies with the type of cartilage, the method of isolation and the age of the animal. The protein moiety of the complex, which accounts for 7 - 8 per cent of the total weight [Hascall & Sajdera, 1970; Rosenberg et al., 1970b], has an amino acid composition characterised by a high proportion of acidic residues and does not contain hydroxyproline. However, it is still uncertain whether all core proteins in a single tissue are absolutely identical [Serafini-Fracassini

et al., 1967; Tsiganos & Muir, 1969; Tsiganos et al., 1971; Heinegard, 1972a].

It should also be noted that preparations obtained from many sources exhibit considerable macro-heterogeneity in their chemical compositions. This is indicated by a certain degree of variability in both the protein content and the galactosamine to glucosamine ratio of their constituent macromolecules [Hoffman et al., 1967; Muir & Jacobs, 1967; Brandt & Muir, 1969; Tsiganos & Muir, 1969; Brandt & Muir, 1971a; Brandt & Muir, 1971b; Tsiganos et al., 1971; Šimůnek & Muir, 1972; Heinegard, 1972b]. It is generally accepted that differences in the galactosamine to glucosamine ratio show that the number of keratan sulphate chains bound to each core in a population of macromolecules is not constant. Variability in the protein content may reflect either differences in the number of identical chondroitin sulphate chains present in each complex [Hascall & Sajdera, 1970; Tsiganos et al., 1971; Hranisavljević et al., 1972; Kao et al., 1972; Šimůnek & Muir, 1972] or

polydispersity of the glycosaminoglycan moiety [Bentley & Rokosova, 1970; Brandt & Muir, 1971a]. In relation to the latter possibility it may be noted that the chain length of chondroitin sulphate can exhibit regional differences in some matrices [Loewi, 1953; Hjertquist & Engfeldt, 1967; Wasteson, 1971; Hjertquist & Wasteson, 1972; Wasteson et al., 1972].

At the time when Mathews and Lozaityte's model was proposed, it was observed that proteoglycan macromolecules were liable to undergo aggregation under conditions that minimised electrostatic repulsion. Therefore, in subsequent studies efforts have always been made to identify the smallest macromolecules which could participate in such an aggregation process and also to ensure that macromolecular degradation was not induced by the extraction procedure. The latter requirement appears to be met by extraction methods based on gentle stirring of the tissue fragments with solvents of high ionic strength, such as 2M-calcium chloride or 4M-guanidinium

chloride, which have been shown to remove up to 80 per cent of the total proteoglycans from bovine nasal cartilage [Sajdera & Hascall, 1969]. Using these so-called dissociative techniques, followed by either fractionation with neutral salts or separation on a caesium chloride density gradient, two preparations, which have been designed PP-L3 [Rosenberg et al., 1970b] and PGS [Hascall & Sajdera, 1970], have been characterised. Their molecular weights have been quoted as 2.4×10^6 and 3.5×10^6 daltons respectively, when identical values were taken for their partial specific volumes [Rosenberg et al., 1970b]. A critical discussion on the composition and physico-chemical parameters of these two preparations has been published and it is suggested that PP-L3 represents the smallest proteoglycan unit in bovine nasal cartilage [Rosenberg et al., 1970b]. However, these macromolecular complexes exhibited such a large degree of poly-dispersity that it may be that too great a significance has been attached to data which represent averages

of widely spread values. Thus, in a PGS preparation the molecular weight was reported to be 2.5×10^6 with a standard deviation of 1.16×10^6 [Hascall & Sajdera, 1970]. Furthermore, these molecular weights have been derived from sedementation velocity and intrinsic viscosity experiments and require for their evaluation: (a) two non-linear extrapolations of experimental data to vanishing concentration, and (b) utilisation of mathematical relationships which do not necessarily apply to the system under investigation and which require assumptions to be made as to the shape of the macromolecules [Hascall & Sajdera, 1970; Woodward et al., 1972]. Bovine nasal cartilage PP-L3 analysed by equilibrium centrifugation, to which the above-mentioned ambiguities do not apply, exhibited whole-cell average molecular weights ranging from 1.1×10^6 ($\overline{M}_w$) to 1.2×10^6 ($\overline{M}_z$), which are about half the value obtained from sedimentation velocity experiments [Wells & Serafini-Fracassini, 1973].

However, there is some evidence which suggests

that even a macromolecule of this size may not be the minimal unit of proteoglycan. On the contrary, it may be the end-product of an in vivo aggregation of smaller subunits. Presumptive evidence for the existence of a self-associating system of nasal cartilage proteoglycan macromolecules during equilibrium centrifugation has been reported [Wells & Serafini-Fracassini, 1973]. In addition, treatment of this proteoglycan with a non-ionic detergent has been shown to produce a marked decrease in the average molecular weight of the complex with minimal or monomer $\bar{M}_n$ lower than 2.4×10^5 [Wells & Serafini-Fracassini, 1973]. This evidence is consistent with the hypothesis that proteoglycan polydispersity may arise as the emergent property of an associating system of subunits in which hydrophobic bonds might be operative. Although the protein core was found lacking in any secondary structure [Eyring & Yang, 1968], binding of the polypeptide cores of subunits to one another may possibly be achieved by the overlap of short sequences of apolar residues in suitable extended conformation.

One could thus postulate that in any given system, including that *in vivo*, the equilibrium state in the aggregating process will be specific to the operative conditions. Hence, considerable caution should be exercised in interpreting the native state of the macromolecular structure from experimental data.

The presence of residual amino acids in heparin extracted by various procedures [Green *et al.*, 1961; Lindahl *et al.*, 1965] strongly suggests that the polysaccharide occurs in the native state covalently bound to a protein moiety. Moreover, the sugar nucleotides normally associated with glycosaminoglycan synthesis have been identified in mast-cell tumours, so it can be assumed that synthesis of these glycosaminoglycans follows a similar scheme [Silbert, 1963,1970]. The synthesis of heparin and other glycosaminoglycans requires the presence of a specific protein backbone which may be described as a biological primer, and as previously indicated the presence of such a moiety may be

associated with the molecular organisation of these complexes.

Some controversy still exists about the presence of multichain proteoheparin in mast-cell granules. The only evidence so far reported is the demonstration within these organelles of beaded structures 35Å in diameter which have been interpreted as proteoheparin [Serafini-Fracassini et al., 1969a]. Particles were seen to be located in pairs or aggregates, suggesting that the complex could exist in a doublet form. Chemical and physical chemical analysis of the extracted heparin have shown these macromolecules to contain 13 per cent protein and have a molecular weight of about 20,000. Chain weights estimated from xylose content gave values in the order of 11,000 daltons, thus suggesting the presence of two heparin chains attached to a polypeptide core [Serafini-Fracassini et al., 1970a]. Contrary to these findings, Lindahl (1970) has identified single protein-free heparin chains ($\bar{M}_W$ 7.4 x 10^3) as the major heparin species present in bovine liver capsule.

In order to clarify this position the aim of this thesis was to:

(1) Develop an extraction procedure for the isolation and purification of proteoheparin from bovine liver capsule which would minimise physical macromolecular disruption.

(2) Chemically and physically characterise the individual proteoheparin species present in this tissue.

Due to the low content of heparin in various anatomical locations large quantities of tissue have to be processed in order to obtain any appreciable quantity of glycosaminoglycan. This gives rise to serious problems both during extraction and purification. The ideal answer is to apply a non-disruptive extraction procedure and then to selectively precipitate the glycosaminoglycan with a specific reagent which would not cause structural damage. Most modern extraction procedures, especially those on an industrial scale, unfortunately still follow the method of Charles and Scott (1933)

in that the tissue is treated with both alkali and endoproteases [Gardell, 1952; Jaques & Bell, 1959; Scott, 1960], which may damage the native compound.

Nucleic acids from bacterial sources were first shown to be selectively precipitated by the use of cetyltrimethylammonium bromide [Jones, 1953] and Scott *et al.*, (1957), were able to apply this procedure to the fractionation of heparin. Adaptation of this method, using the cationic detergent cetylpyridinium chloride in the fractionation of extracted polyanionic substances [Scott, 1960; Schiller *et al.*, 1961], can be termed the 'critical electrolyte concentration procedure'.

This reaction is based on the ion-exchange equation:

$$P^{z-}\ zM^{+} \ + \ zR^{+} \ \leftrightharpoons \ P^{z-}\ zR^{+} \ + \ zM^{+}$$

in which P is a polymer with z negative charges, R^{+} is an organic cation and M^{+} an inorganic counterion. The extent of the change from reactant to product is:-

$$B = \frac{[P^{z-}\ zR^+]}{[P^{z-}\ zM^+]} = K \left[\frac{R^+}{M^+}\right]^z$$

where K is the equilibrium constant.

If $[R^+]$ is kept constant by using the organic cation in relatively high concentration and z is large as in some bio-polymers, B is highly dependent on $[M^+]$ the concentration of the inorganic cation, and changes from large to very small values over a narrow range of $[M^+]$ especially if the product $P^{z-}\ zR^+$ is an insoluble precipitate. The critical electrolyte concentration at which this large change in B occurs is influenced by the value of K which is determined by the relative affinities of M^+ and R^+ for the negatively charged groups of the polyanion, and it therefore differs not only with the nature of M^+ and R^+ but with the nature of anionic groups, which in mammalian tissues may be COO^-, OSO_3^- or PO_4^-. Thus for any R^+/M^+ pair, the binding of R^+ by P^{z-} is suddenly reduced practically to zero at the critical electrolyte concentration which is dependent on the nature of the polymer.

Serafini-Fracassini et al. (1969b) reported the extraction and precipitation of a heparin-protein complex using cetylpyridinium chloride; however, this method of extraction has been criticised on the grounds that the protein moiety associated with the complex might have been an impurity which had resulted from the binding of acidic proteins to cetylpyridinium chloride [Lindahl, 1969]. It is interesting to point out that repeated cetylpyridinium chloride precipitation of a PP-L fraction isolated from bovine nasal cartilage showed a marked decrease in protein content [Serafini-Fracassini et al., 1967], therefore such a criticism should be regarded as invalid.

However, in view of the above difficulties it was decided to develop an extraction procedure which avoided the use of cetylpyridinium chloride, and it was found that utilisation of dissociative solvents was effective on liver tissue. The subsequent purification steps involved ion exchange chromatography.

METHODS and MATERIALS

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Chemical Procedures

Hexuronic acid was measured directly by the method of Bitter and Muir (1962), using glucurono-lactone as the standard.

In order to determine hexosamine content, samples of known weight (approx. 1 mg.) were, firstly, dissolved in 2 ml. of 4M-HCl and hydrolysed under nitrogen in sealed tubes at 110°C for 8 h. The following methods were then used:

(a) After hydrolysis, excess acid was neutralised and total hexosamines determined by the Elson and Morgan (1933) reaction, using the distillation procedure of Cessi and Piliego (1960).

(b) Differential determination of glucosamine and galactosamine was achieved on a Locarte amino acid analyser.

(c) The gas liquid chromatography procedure of Stimson (1970) was also used. Briefly, the hydrolysed samples were dried under vacuo and the

trimethylsilylated derivative was formed using bis(trimethylsilyl)trifluoro-acetamide (BSFA). Both glucosamine and galactosamine can be estimated by this method.

The amount of N-sulphated hexosamine was determined according to the methods of Dische and Borenfreund (1950), and Lagunoff and Warren (1962).

Neutral sugars were identified and quantitatively estimated by gas chromatography [Sweeley et al., 1963].

At many stages in this project quantities available for a complete sugar analysis were rather small. In order to obtain a spectrum of the sugars present in a preparation, the method of Bhatti et al., (1970) was adopted. Even sugars such as N-acetylglucosamine and iduronic acid can in fact be detected and estimated quantitatively by this technique.

Individual xylose and galactose values were also determined by colorimetric procedures [Tsiganos and Muir, 1966; Lyons and Singer, 1971].

Protein concentrations were obtained from amino

acid analysis data and in one instance by the micro-biuret method [Itazahaki and Gill, 1964], with serum albumin as a standard.

Samples for amino acid analysis were hydrolysed in constant-boiling HCl (2 ml. per mg. of material) under vacuo at 110°C for varying intervals of time. Prior to evacuating the hydrolysis tubes, the acid solutions were gassed with nitrogen. The samples were taken down to dryness in a rotary film evaporator, the temperature of the bath being at 30°C. Amino acid analyses were carried out on a Locarte amino acid analyser. Tryptophan was not analysed in view of its doubtful occurrence in heparin [Serafini-Fracassini et al., 1969b].

N-terminal analysis was carried out by the dansylation procedure as described by Woods and Wang (1967). Dansylated amino acids were separated by chromatography on polyacrylamide sheets (4 cm^2) using a three solvent system.

Sulphur contents were determined by two procedures, colorimetrically by the method of Antonopoulos (1962),

and by gas chromatography [Srinivasan et al., 1970].

Electrophoresis

Two types of electrophoresis were adopted.
(1) Disc gel electrophoresis was run on a Shandon apparatus according to the procedure of Ornstein and Davis (1964).
(2) Paper electrophoresis: all chromatography paper prior to use was washed with 5 per cent glacial acetic acid for one week, dried, and then stored in an enclosed container. High-voltage electrophoresis was performed at -5°C on strips (35 x 58 cm) of Whatman 3MM paper, using a Pherograph Original Frankfurt apparatus. A 1.2M-pyridine — 0.17M-acetic acid (pH 6.0) buffer was used. Papers were stained with ninhydrin or by a toluidine blue dip procedure. This method involved washing the paper in EtOH-ether (95:5) and staining with 0.06 per cent toluidine blue in 0.5 per cent acetic acid. Excess dye was removed by carefully rinsing the paper with 2 per cent acetic acid [Dawson et al., 1969]. Commercial

heparin (Wilson Laboratories Lot No.136651) was used as a standard.

Ultracentrifugation

Ultracentrifugation was performed on a Spinco Model E Ultracentrifuge. All experiments were carried out at 20°C in an Anil rotor.

The method of Chervenka (1970) was followed; this technique involves the layering of a relatively large volume of solvent on to a sample solution in a capillary synthetic boundary cell. The presence of solute in the upper part of the cell increases the resolution at the meniscus.

The samples (approx. 600µg.per ml.) were dialysed exhaustively against 2 per cent KCl at 4°C and 0.05 ml. of these solutions were placed in the sample sector of a 12 mm. double-sector capillary boundary cell. The reference cell was filled with 0.45 ml. of the dialysate. It was found that equilibrium was obtained after twenty hours; even so plates were taken at hourly intervals for comparison

purposes. The criterion for depletion was taken as the absence of fringe displacement for at least one third of the column height. A water blank was run to correct for optical distortions.

Results were plotted in the form of log(Yr-Yo) [Yr-Yo is the net fringe displacement], against r^2 [r is the distance of each Yr point from the centre of rotation].

Values for the point average effective reduced molecular weights were defined as $\alpha w(r)$ [$\alpha w(r) = M\left(\frac{1 - \bar{v}P}{2RT}\right) w^2$] were plotted against r^2. Where $\bar{v}$ is the partial specific volume of the solute, P is the density of the solvent, R is the gas constant, w the angular velocity, T is the absolute temperature and M the apparent molecular weight.

These values were derived by determination, via least mean squares, of the slope of the straight line through successive groups of five points. $\bar{M}z$ over the whole cell was obtained by extrapolation of $\alpha w(r)$ values to the base of the column. Similarly $\bar{M}w \rightarrow o$

values were obtained by extrapolation to the meniscus.

Determination of Partial Specific Volume

Apparent partial specific volumes were estimated from density measurements carried out in a density column, prepared from mixtures of bromobenzene (freshly distilled) and kerosene, to give a gradient over the range of 0.99 - 1.03 s.g. [Hvidt et al., 1954; Miller and Gasek, 1960]. Drops of 0.5 µl. were applied and the equilibrium positions were determined using a cathetometer. It was found that slight changes in drop size made no detectable difference to the equilibrium position. The column was enclosed in a water jacket maintained at 20.00 ± 0.01°C. The apparent specific volume was calculated from the relation:-

$$\bar{v} = \frac{100/d - (100-n)/d_o}{n}$$

Where n is the concentration in g. per 100 ml and d is given by:-

$$d = d_1 + \frac{(h - h_1).(d_2 - d_1)}{(h_2 - h_1)}$$

d is the density of the solution

d_o is the density of the solvent

d_1 is the density of a sucrose standard and h_1 its position.

d_2 is the density of the next standard.

The column was calibrated using solutions of sucrose of known density.

Since glycosaminoglycans are polyelectrolytes with high charge to weight ratios, it is necessary to reduce the effects of non-ideality. Partial specific volumes were estimated as recommended by Casassa and Eisenberg (1964) after exhaustive dialysis against solvent.

Infrared Spectroscopy

Spectra were obtained in a Perkin-Elmer Model 237B grating infrared spectrometer with a NaCl prism.

Approximately 1.5 mg. of sample was ground with 150 mg. of KBr.

Electron Microscopy

Approximately 6 μg. per ml. solution of H2b was firstly dialysed against water for two days at 4°C. The bismuth nitrate stain was prepared by dissolving 1.0 g. of bismuth nitrate in 10 ml. of 2M-nitric acid and then making up to 200 ml. with water, giving an 0.5 per cent solution in 0.1M-nitric acid. The sample was sprayed on to carbon filmed grids and dried. The grids were stained for 15 min., washed successfully with 2M-nitric acid, water, and finally dried [Serafini-Fracassini et al., 1969a].

Extraction and Isolation Procedure

Livers from freshly killed oxen were obtained from a local abattoir and the capsules were quickly stripped free from tissue debris. The capsules were partially defatted in acetone, ground and stored at 4°C.

Initially the tissue was extracted with 4M-guanidinium chloride; however, on removal of the guanidino, either by dialysis or micro-filtration, a large insoluble fraction was formed which contained hexuronic acid. This indicated binding of glycosaminoglycans to contaminating proteins in the insoluble residue. Similarly, ethanol fractionation of a guanidinium extract showed that the majority of the hexuronic acid-containing material was present in a fraction which precipitated after the addition of only two volumes of ethanol. One could conceivably suggest that on rupture of the mast cell granules the released heparin could associate with proteins, especially those which possess an overall positive charge. Interactions of this type involving glycosaminoglycans and collagen have been clearly illustrated by electron microscopy [Serafini-Fracassini et al., 1970b]. In order to study this type of reaction the following in vitro experiments were followed.

The extraction of insoluble collagen was carried

out according to the method of Gross and Kirk (1958). A cow hide from a four-year-old animal was firstly soaked in a 5% NH_4Cl - 1% NaCl - 1-10,000 merthiolate solution for three days at room temperature. The hair, epidermis and subcutaneous tissue were scraped off and the pure white dermis was cut into fine pieces and then ground down to a powdered form. The tissue was now extracted with 10 volumes of 0.5M-acetic acid for 18 h. at 4°C and the residue was collected by centrifugation and re-extracted with acetic acid for a further three cycles. The tropocollagen-free residue was now dried and stored over P_2O_5.

Insoluble collagen (1 g.) was suspended in a 20 ml. solution of heparin (10 mg.) with varying salt concentrations and pH values. The suspension was gently stirred at 4°C for 3 h. and the supernatant was tested for heparin content.

Results

Solvent	pH	Heparin (% recovery)
Water	-	0
0.1M-$MgCl_2$ to 3M-$MgCl_2$	5	100
0.1M-$MgCl_2$	3	100
0.16M-$MgCl_2$ to 0.25M-$MgCl_2$	3	0

It seems evident from the above results that both pH and electrolyte concentrations are critical factors in the binding of heparin to insoluble protein. This suggests that soluble proteins could easily be removed by extracting the liver tissue with 0.1M salt solution at pH 3.0, without fear of losing mucopolysaccharides which seem to associate with the insoluble residue under these conditions. On the basis of these results, the following procedure was adopted.

Portions (50 g.) of liver capsule were twice extracted with 15 volumes of 0.1M-citrate buffer, pH 3.0, for 24 h. The supernatants were removed

by centrifugation at 12,000 rpm, and no hexuronic acid-containing material was found to be present in the soluble extracts. The residues were evenly dispersed in 15 volumes of unbuffered 3M-$MgCl_2$ and the suspension extracted by gentle agitation for 48 h. The supernatant was collected as above and dialysed exhaustively against water for three days; during this time a precipitate was formed which was subsequently collected and stored (H1). Three volumes of ethanol and one volume of ethanol saturated with K-acetate were added to the supernatant and the precipitated material was collected (H2). The entire isolation procedure was carried out at 4°C in order to minimise the possible effects of protease and heparinase activities [Benditt and Arase, 1959; Lagunoff and Benditt, 1963; Jaques and Cho, 1954].

Fractions H1 and H2 were dissolved in 0.4M-citrate, pH 5.0, and chromatographed on a Whatman DE-52 cellulose column (2 x 8 cm.) by stepwise elution with 0.1M-citrate, pH 4.0, containing varying concentrations of NaCl (0.25M increments were used).

Fractions (4 ml.) were tested for hexuronic acid, with commercial heparin (Wilson Laboratories Lot No.136651) being used as a standard. In the case of H1, hexuronic acid-containing material was present only in the 0.75M eluate. On the other hand, H2 yielded two hexuronic acid-containing fractions which were eluted at 0.5M-NaCl (Fraction H2a) and at 0.75M-NaCl (Fraction H2b).

The three fractions were subsequently dialysed against 0.01M-citrate, pH 5.0 for two days, and chromatographed on a Sephadex G-50 column (2 x 12 cm.) at a rate of 10 ml. per h. The materials not retained by the gel were collected and dialysed firstly against 2 per cent KCl, and then water, before being precipitated by the addition of ethanol and K-acetate at 4°C. The precipitates were collected and dried under acetone and ether. The homogeneity of the preparations was checked in terms of molecular size by gel filtration on a Sephadex G-200 column (2.5 x 50 cm.) in 2M-KCl. Samples (20 mg. in 2 ml.) were eluted at a rate of 8 ml.

per h., and fractions (4 ml.) were collected and tested for hexuronic acid content. H1 and H2b behaved as single monodispersed species. On the other hand, H2a, as illustrated in figure 4, demonstrated the presence of at least three subfractions. Two of these had Glc NH_2 : Gal NH_2 ratios of 0.25 and 0.17, which indicated the presence in fraction H2a of galactosamine-containing glycosaminoglycans in addition to heparin. The third subfraction had a Glc NH_2 : Gal NH_2 ratio of 8.2 and will be referred to as H2a^{o}.

The yields of H1, H2a^{o} and H2b were estimated to be approximately 15, 45 and 85 mg. per 100 g. of dry tissue, respectively, although losses might have occurred during fractionation.

Biological Activity

The biological activity of each fraction was estimated by two procedures [Sharp et al., 1961; Hardistry and Ingram, 1965]. Both techniques involve titrating known quantities of sample against protamine sulphate.

Digestion with Proteolytic Enzymes

Fraction H2b was treated with two proteolytic enzymes, papain (EC 3.4.4.10) and pronase.

Samples (5 mg.) were digested with papain (200 µg) in 0.05M-sodium phosphate - 0-2M-dithiothreitol - 30mM-EDTA buffer, pH 6.0, at 65°C for 48 h [Shulman and Meyer, 1970] and similarly with the same quantity of pronase in 0.001M-$CaCl_2$ - 0.05M-Tris buffer, pH 7.6, at room temperature for 24 h. [Anderson et al., 1965]. The digestion products from both experiments were dialysed against 0.4M-sodium phosphate, pH 5.0, for 18 h. at 4°C, and subsequently chromatographed on a Whatman DE-52 cellulose column (1.5 x 4 cm.). When absorption at 230 nm. ceased the column was washed with 0.75M-KCl in 0.1M-sodium phosphate, pH 4.0, and the eluted fractions (3 ml.) were tested for hexuronic acid. Heparin-containing fractions were now dialysed against 2 per cent KCl, and molecular weights determined by ultracentrifugation.

β-elimination

(a) A 1 mg. per ml. solution of H2b was treated with an equal volume of M-NaOH at room temperature for 24 h. The formation of unsaturated derivatives of the hydroxy amino acids was monitored by measuring the change in optical density at 241 nm. as suggested by Riley et al. (1957). The reaction was compared with that of an identical solution containing PP-L isolated from bovine nasal cartilage. Molecular weights of the degradation products were determined by ultracentrifugation.

(b) The protein moiety isolated from fraction H2b by heparinase digestion was β-eliminated according to the method of Simpson et al. (1972). Material (about 200 µg.) was dissolved in 2 ml. of 0.2M-Na_2SO_3 and the pH adjusted to 11.9. The solution was stirred under nitrogen at room temperature for 24 h. The volume was now reduced by rotary film evaporation and the sample desalted on a Sephadex G-10 column (1.5 x 30 cm.).

Degradation with Nitrous Acid

Fraction H2b was degraded according to the method of Cifonelli (1968). The heparin preparation (9 mg.) was dissolved in water (2 ml.) and the solution passed through a column (1 x 6 cm.) of Dowex 50-X8 (H^+, 200-400 mesh), at 4°C, and followed by a water wash. The combined effluents (16 ml.) were mixed with 12 ml. of glyme (ethylene glycol dimethyl ether) and the solution was cooled to -15°C. 0.1M-nitrous acid (9 ml.) in 60 per cent glyme, which had been freshly prepared [Scanley, 1963], was added and deamination was allowed to proceed in the dark. After 12 h., 0.5 ml. of 12.5 per cent ammonium sulphamate were added and the mixture was placed in the cold (4°C) for 1 h., with occasional shaking. The solution was neutralised with NaOH, concentrated to about 3 ml. and desalted on a Sephadex G-10 column (1.5 x 30 cm.). Fractionation of the acid degraded products was achieved by a two-step procedure:-

(1) The salt free solution was twice chromatographed on a Whatman DE-52 cellulose column (2 x 6 cm.) at a rate of 20 ml. per hour. The eluted material was further fractionated by electrophoresis.

(2) Concentrated samples (0.5 ml.) were applied to Whatman 3MM paper in 0.175M-acetic acid — 1.2M-pyridine, pH 6.0, for 2 h., at 80mA and 1000V. Ninhydrin and toluidine blue positive areas were eluted from the paper with water.

Degradation with Heparinase

a) Extraction of Heparinase from Flavobacterium heparinum (ATCC 13126)

The bacteria were grown and harvested according to Payza and Korn (1956) and Linker and Hovingh (1965).

A 10 litre culture of F. heparinum after inoculation with a 150 ml. 24 h. culture of cells was grown in 2.75 per cent (w/v) trypticase soy broth without dextrose (B.B.L. Ltd.) in the presence of 0.01 per cent (w/v) heparin (Sigma Ltd.) in a

New Brunswick MF 114 fermentor at 24°C with an aeration rate of 10 litres per minute. The level of heparin in the medium was monitored at regular intervals by the procedure of Jaques et al. (1949), the rate of heparin consumption being shown in figure 5. After 24 h. the heparin level had dropped to 0.06 mg. per cent, and at this point the bacteria were harvested, using a continuous head at 18,000 rpm. Some losses were evident since the supernatant was not perfectly clear, this being due to the extremely small size of the micro-organisms. No heparinase activity was found in the supernatant on assaying a concentrated sample.

The cells were washed in 0.035M-phosphate buffer, pH 7.0, suspended in a small volume of the same buffer, and sonically disrupted for 10 minutes in a Brownwill sonicator at 2°C. The cell debris was centrifuged off at 18,000 rpm and the supernatant was then lyophilised. Approximately 44 per cent of the dried material was estimated to be protein by the micro-biuret method.

b) Assay of heparinase activity:

Incubations were carried out at 25°C in 0.1M-sodium acetate, pH 7.4, with substrate concentrations of 10 mg. per ml. and the enzyme at 0.5 to 1.0 mg. per ml. (insoluble protein when present was removed by centrifugation). The activity of all preparations was assayed by measuring the increase in reducing sugar content [Marais et al., 1966]; glucosamine was used as a standard.

c) Fractionation of the crude heparinase

The fractionation of the heparinase complex into various enzymic components, i.e. eliminase, glycuronidase and sulphatases, was obtained by the method adopted by Hovingh and Linker (1970).

Crude lyophilised extract of Flavobacterium (6 g.) was added to an 0.5M-sodium acetate solution (80 ml.), and the pH was adjusted to 6.4. The suspension was stirred overnight at 4°C and the insoluble residue removed by centrifugation. The

soluble extract was treated with protamine sulphate (10 mg. per ml. of solution), stirred for 3 h. in the cold (4°C), and the precipitated material was removed as above. The solution was now diluted to an 0.05M concentration of acetate and added to a Whatman P.11 phosphocellulose column (2.5 x 20 cm.) which had been previously charged in 0.5M-HCl. The column was washed with 0.025M-Tris, pH 7.9, in 0.1M-NaCl until the effluent was free from absorption at 230 nm. A gradient was formed by the addition of 500 ml. of 0.025M-Tris in 0.7M-NaCl, pH 7.9, to a mixing vessel containing an equal volume of 0.025M-Tris in 0.1M-NaCl, pH 7.9. Fractions, (6 ml.), were collected at a rate of 60 ml. per hour and were assayed for protein and heparinase activity (figure 6).

Fractions in the main peak (80 - 100 ml.) were pooled, and subsequently dialysed against an excess of water for two days, and then against 0.01M-sodium acetate, pH 7.9, overnight. The active fraction was

re-fractionated on phosphocellulose (1 x 12 cm.). The column was first eluted with 0.025M-sodium acetate in 0.075M-NaCl and then with 0.025M-sodium acetate in 0.175M-NaCl. The majority of the activity was found in the second eluate, which was dialysed against an excess of $(NH_4)_2CO_3$, pH 7.5, and lyophilised. All purification steps were carried out at 4°C.

The purity of heparinase (eliminase) was checked by disc gel electrophoresis which showed the presence of two strong bands and at least three weaker bands.

d) Digestion procedure and fractionation of degradation products

Lyophilised enzyme preparation (1 mg.) was dissolved in 10 ml. of 0.1M-sodium acetate, pH 7.0; H2b (20 mg.) was now dissolved in 2.5 ml. of this solution and incubated at 31°C for 12 h. The hydrolysis products were chromatographed on a

Whatman DE-52 cellulose column (2 x 8 cm.) which had been equilibrated in water. The eluted material was collected and desalted on Sephadex G-10. Individual fractions were isolated and identified by high voltage electrophoresis on Whatman 3MM paper and by staining with ninhydrin and toluidine blue. The ninhydrin positive area was eluted with water, dried and stored under vacuo.

Figure 4

The elution profile of fraction H2a on a Sephadex G-200 column (2.5 x 50 cm.), void volume 75 ml., in 2M-KCl at a rate of 8 ml. per h. Effluent fractions (4 ml.) were analysed for hexuronic acid content by the carbazole reaction. Glucosamine and galactosamine values for the three sub-fractions were determined by the ninhydrin after separation on ion exchange.

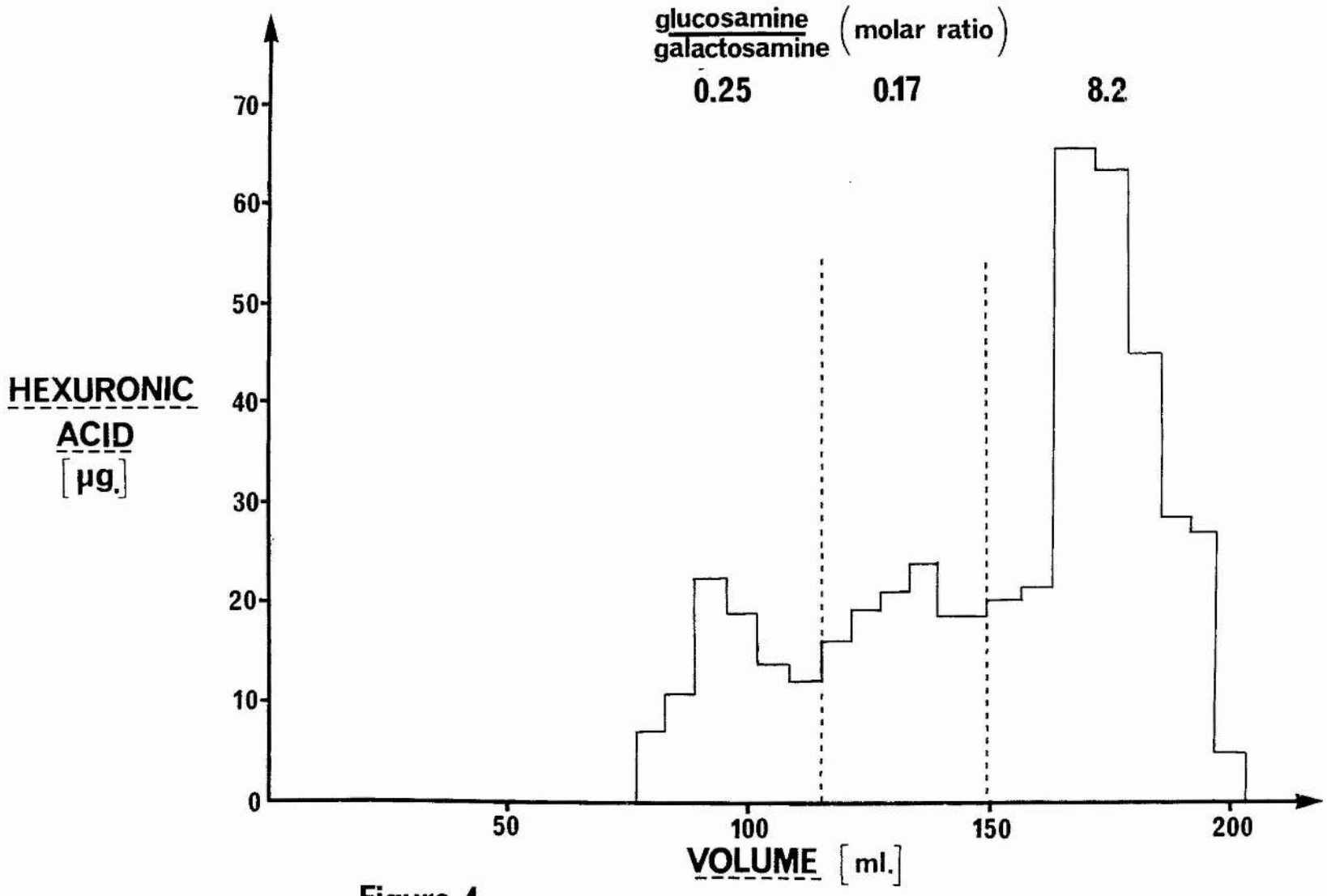

glucosamine / galactosamine (molar ratio)
0.25
0.17
8.2
HEXURONIC ACID [µg.]
70
60
50
40
30
20
10
0
50
100
150
200
VOLUME [ml.]

Figure 4

Figure 5

The growth of a heparin induced F. heparinum culture grown in a New Brunswick M.F.114 fermentor at 24°C. with an aeration rate of 10 litres per min. The rate of heparin consumption with time was routinely followed by measuring the decrease in metachromasia of azure A.

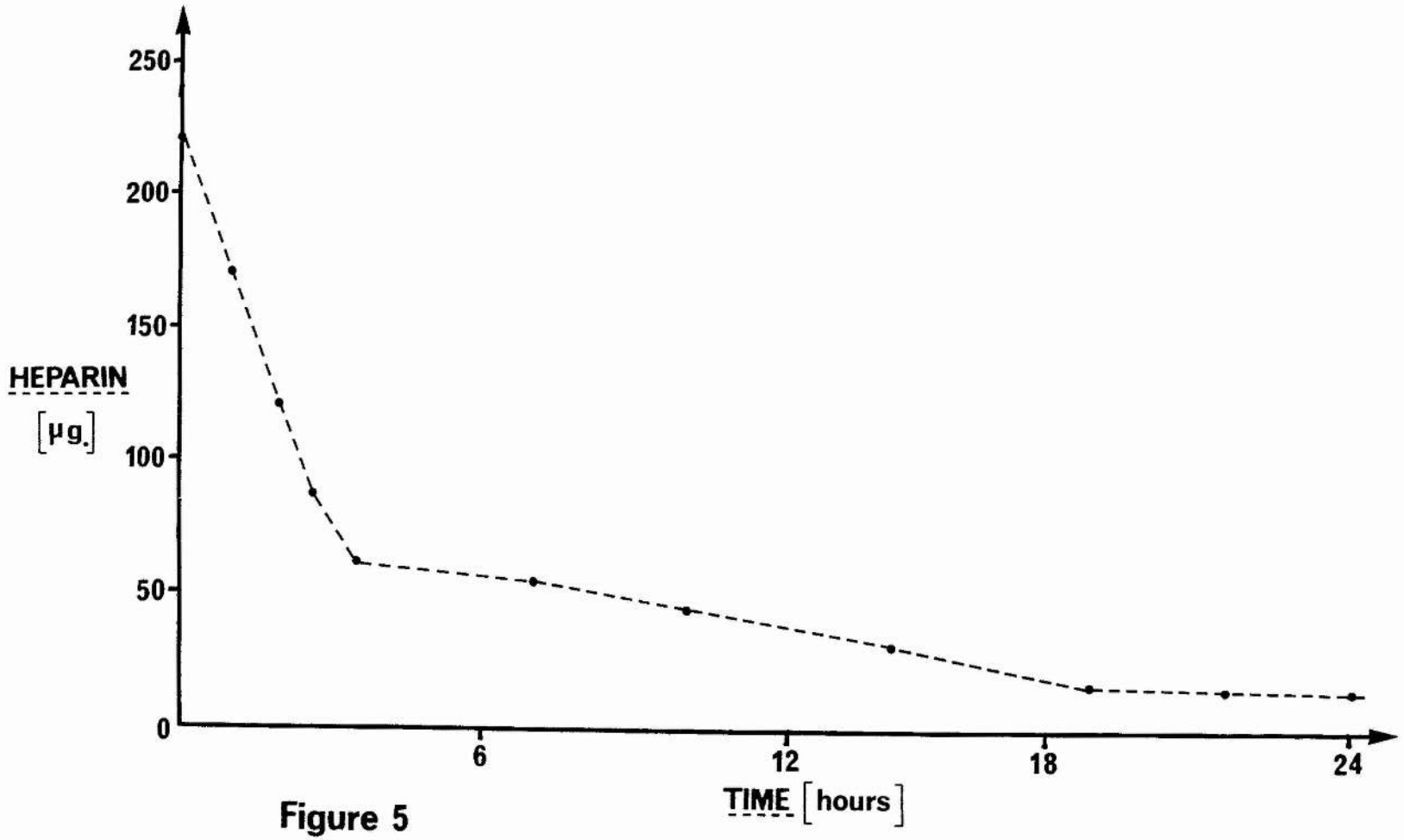


Figure 5

Figure 6

Elution profile of heparinase from a large phosphocellulose column (2.5 x 20 cm.), fractions (6 ml.) were collected at a rate of 60 ml. per h. Heparinase activity (———) was determined by incubating aliquots (0.5 ml.) at 25°C. with heparin as substrate in 0.1M sodium acetate, pH 7.4 for 2 h., and then measuring the reducing sugar content. The elution of protein (—•—•—) was obtained by measuring absorption at 280nm; (------) corresponds to the NaCl gradient.

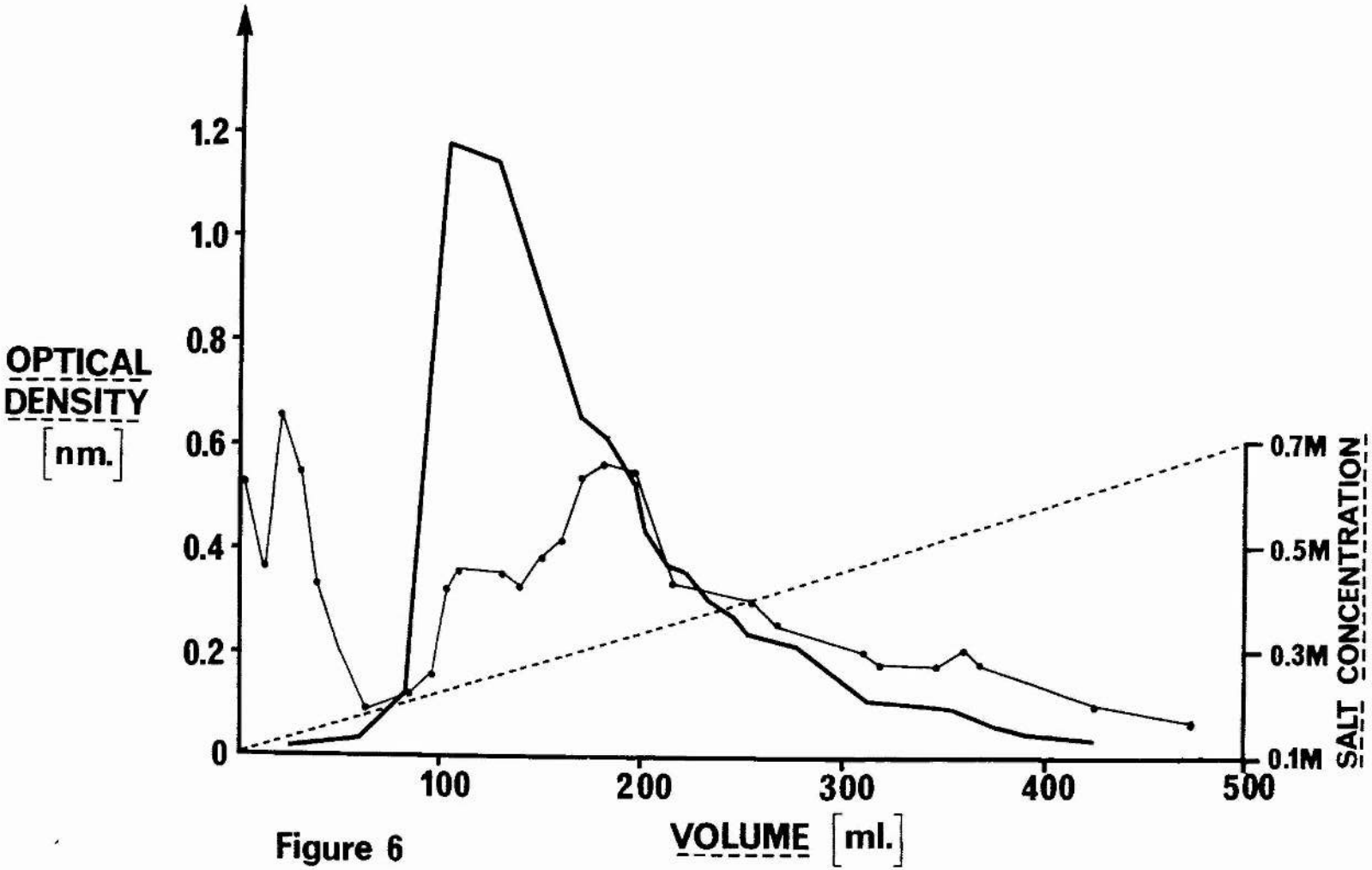

OPTICAL DENSITY [nm.]
1.2
1.0
0.8
0.6
0.4
0.2
0
100
200
300
400
500
VOLUME [ml.]
0.7M
0.5M
0.3M
0.1M
SALT CONCENTRATION

Figure 6

RESULTS

RESULTS

The chemical compositions of the three heparin species isolated from liver capsule are illustrated in Table I. A generally accepted criterion of purity as far as heparin is concerned is the lack of galactosamine in the preparation. However some galactosamine appears to be present in all analyses reported in the literature in which separation of the two isomers was carried out [Lindahl *et al.*, 1965; Lindahl and Rodén, 1965; Lindahl, 1966; Serafini-Fracassini *et al.*, 1969b; Lindahl, 1970; Linker and Hovingh, 1972], and possibly this amino sugar may represent a minor constituent of the macromolecule. In keeping with this view, fraction H2b was found to contain about 3 per cent galactosamine after repeated ion exchange chromatography. However, galactosamine was not found to be present in fraction H1 and consequently the previous assumption must be regarded with some caution.

The heparin species obtained as fraction $H2a^{o}$ were found to contain at least 12 per cent galactosamine

TABLE I

Composition of the various heparin species isolated from bovine liver capsule. All individual values are expressed as percentages of dry and ash-free material.

TABLE I

Composition of the proteoheparin species

	H1		H2a°		H2b	
Total hexosamine (as free base)		20.0		23.1		18.9
Glucosamine	20.0		20.6		18.4	
Galactosamine	0.0		2.5		0.5	
Hexuronic acid (as free acid)	24.8		28.6		29.4	
Hexuronic acid (as potassium salt)		29.7		34.3		35.4
Sulphur (as S)	9.2		7.8		8.0	
Sulphur (as ester sulphate, potassium salt)		34.9		29.5		30.7
Galactose		3.1		3.5		2.9
Xylose		1.7		1.8		1.4
Protein		10.7		7.5		10.7
Total		100.1		99.7		100.0
Sulphate:Glucosamine (molar ratio)		2.6		2.2		2.5

The other two components present in the parent fraction H2a, which exhibited glucosamine : galactosamine ratios of 0.17 and 0.25 respectively were isolated and analysed. Preliminary characterisation studies have shown so far that they contain iduronic acid, and therefore heparin H2a^{0} may be contaminated by some dermatan sulphate which has been detected in bovine liver capsule [Lindahl, 1970].

The hexuronate : glucosamine molar ratio should be equal to unity; however in all preparations this ratio was found to be greater than the theoretical values. This is in agreement with data reported where the ratio ranges from 1.1 to 2.0 [Lindahl *et al.*, 1965; Lasker and Stivala, 1966; Serafini-Fracassini *et al.*, 1969b; Hovingh and Linker, 1970; Lindahl, 1970; Ögren and Lindahl, 1971; Hirano, 1972]. It has recently been suggested [Linker and Hovingh, 1972] that determination of hexosamines by the Elson and Morgan reaction could yield low values; however, in our experience the hexosamine content determined by the distillation procedure of Cossi

and Pilliego (1960) was always in agreement with the results obtained by the ninhydrin reaction. All preparations were found to contain N-sulphated glucosamine units, this being a further indication that the isolated glycosaminoglycans consist of heparin.

The degree of sulphation estimated for each fraction was found to be in keeping with its chromatographic properties, namely H2a^{0}, which was eluted from Whatman DE-52 cellulose at a low electrolyte concentration, . . exhibited a correspondingly lower sulphate content than fractions H1 and H2b. Ion exchange chromatography should ensure that the sulphate groups estimated are covalently bound to the polysaccharide, and so the possibility of contamination from free sulphate residues can be disregarded. Sulphate : glucosamine molar ratios also suggest that fractions H1 and H2b contain five sulphate groups per tetrasaccharide unit, whereas fraction H2a^{0} only contains on average four sulphates although it must be remembered that this fraction may

contain some low sulphate-containing compounds.

The identification of galactose and xylose in a molar ratio which is in keeping with the reported structure of the linkage region [Lindahl and Rodén, 1965], indicates that the potentially reducing ends of the glycosaminoglycan chains are intact in the tissue.

All heparin species were found to contain appreciable quantities of protein. It seems reasonable to suggest on the basis of the isolation and purification procedures used in this project that the polysaccharide and the protein moiety are covalently bound and in keeping with this hypothesis amino acid analyses revealed in all cases similar amino acid profiles (Table II).

All heparin fractions were found to be biologically active, however this activity did vary according to individual species (Table III). It is rather difficult at present to relate biological activity to any specific chemical or physical property of a heparin macromolecule [Lasker and Stivala, 1966].

TABLE II

Amino acid analysis of the various protecheparin species. The results are expressed in residues of amino acids per 1000 residues. Values were obtained by hydrolysing samples for varying time intervals in order to correct hydrolytic losses.

TABLE II

Amino acid analysis of the individual proteoheparin species

Amino Acid	H1	H2a°	H2b
Hydroxyproline	25.0	0.0	0.0
Aspartic acid	78.9	107.1	72.7
Threonine	25.6	52.6	36.3
Serine	135.1	173.5	181.5
Glutamic acid	85.4	111.7	114.7
Proline	47.2	37.4	36.3
Glycine	263.1	217.7	224.8
Alanine	88.5	79.9	83.7
Valine	44.7	42.5	43.5
Cystine (½)	0.0	0.0	0.0
Methionine	0.0	0.0	0.0
Isoleucine	21.5	18.5	24.5
Leucine	44.0	50.8	71.3
Tyrosine	Trace	Trace	Trace
Phenylalanine	41.0	29.8	30.5
Hydroxyproline	0.0	0.0	0.0
Lysine	50.8	28.0	24.0
Histidine	0.0	18.0	24.0
Arginine	50.5	33.7	32.4

TABLE III

Biological activities were determined by two techniques which involved titrating the samples against protamine sulphate. The protamine sulphate used (1 mg.) was found to be equivalent to 86 B.P. units per mg. lung heparin, and 110 B.P. units per mg. mucous heparin. An average value of 100 B.P. units per mg. was taken for the heparin standard.

TABLE III

Biological activity values of the heparin species present in bovine liver capsule.

	H1	H2a°	H2b
Biological Activity (B.P. units per mg.)	145	110	180

For example, Laurent (1961) correlated biological activity with molecular weight when considering a number of commercial heparin samples, and this was further supported by Ögren and Lindahl (1971) in their study of heparin from mouse mastocytoma. However Barlow et al. (1961) reported the presence of a high molecular weight heparin species with low biological activity. On the other hand the degree of sulphation may also represent an important factor in this respect; however, Lasker and Stivala (1966) found that on treating heparin with acid under mild conditions, biological activity decreased with time of treatment although no loss in sulphur content was observed. This suggests that some form of structural organisation must bear some influence on biological properties. The results obtained for fractions H1, H2b and H2a° indicate that biological activities are affected by both molecular weights and sulphur contents.

Data reported in Table IV show that heparin species present in bovine liver capsule fall into

two classes of macromolecules which exhibit a 1:2 relationship regarding their molecular weights at vanishing concentrations. It should be pointed out that the range of $\bar{M}n$ app obtained at vanishing concentrations and at the base of the column for H1 and H2b which constitute the high-molecular weight species may reflect some degree of macromolecular aggregation. On the other hand, it should be noted that polydispersity of the heparin chains may grossly affect molecular weight estimations since reduction in chain length by only one tetrasaccharide unit would result in a decrease in molecular weight of about 16 per cent when an average chain weight value of 8,000 daltons is assumed.

Whole-cell and C=O averages of fractions H1 and H2b clearly fall into very sharp ranges when compared with the data of a highly purified lysozyme sample (Table IV), thus indicating a high degree of homogeneity of individual heparin species. Furthermore, the molecular weight values of these

TABLE IV

The average molecular weights of the various heparin species were determined by equilibrium ultracentrifugation. Approximately 800µg. per ml. of sample in 2 per cent KCl was centrifuged at a speed of 39460 rev. per min., at 20.0°C, for a minimum of 20 h. Molecular weight was calculated using an experimentally determined partial specific volume value of 0.56 ml $g.^{-1}$ and the density of a 2 per cent KCl solution was found to be 1.0110 $g.ml^{-1}$, at 20.0°C.

In order to compare and assess the variation in the different types of average molecular weights determined, namely $\bar{M}n$, $\bar{M}w$ and $\bar{M}z$, a highly purified sample of lysozyme was analysed under identical experimental conditions. A partial specific volume of 0.75 $ml.g^{-1}$ was taken for the protein sample.

TABLE IV

Sample	Whole-cell		Meniscus			Column base			Chain Weight
	$\bar{M}_{wx}10^{-3}$	$\bar{M}_{zx}10^{-3}$	$\bar{M}_{nx}10^{-3}$	$\bar{M}_{wx}10^{-3}$	$\bar{M}_{zx}10^{-3}$	$\bar{M}_{nx}10^{-3}$	$\bar{M}_{wx}10^{-3}$	$\bar{M}_{zx}10^{-3}$	(Calculated from xylose content)$x10^{-3}$
H1	20.51	22.30	15.5	17.92	19.43	22.00	25.37	27.79	8.0
	$\pm$0.26	$\pm$0.66	$\pm$0.21	$\pm$0.43	$\pm$3.26	$\pm$0.19	$\pm$0.35	$\pm$5.12	
H2a°	14.26	20.17	7.59	9.81	19.10	13.55	19.61	21.28	6.7
	$\pm$0.38	$\pm$0.37	$\pm$0.18	$\pm$0.73	$\pm$2.11	$\pm$0.21	$\pm$0.40	$\pm$2.21	
H2b	19.94	20.77	16.22	16.72	16.76	20.20	21.37	28.89	8.6
	$\pm$0.14	$\pm$0.51	$\pm$0.24	$\pm$0.25	$\pm$2.44	$\pm$0.11	$\pm$0.40	$\pm$2.51	
Lysozyme (highly purified)	13.73	13.93	12.76	13.42	12.60	13.81	13.96	15.46	
	$\pm$0.05	$\pm$0.24	$\pm$0.08	$\pm$0.26	$\pm$1.88	$\pm$0.06	$\pm$0.26	$\pm$1.86	

two fractions showed remarkable similarities. On the other hand, molecular weight averages of fraction H2a° reflect the presence of at least two macromolecular species in this preparation.

The chain weights, calculated from xylose content are similar in all three fractions and an average value of 7,800 daltons can be taken. However, while this value is in keeping with the $\bar{M}_{n_{C=O}}$ for H2a°, one finds that it is only half of those recorded for H1 and H2b. It is also interesting to note that heparin chains isolated from bovine liver tissue by Lindahl (1970) were found to have molecular weights ($\bar{M}_W$) in the region of 7,400 daltons which is in good agreement with the average chain weights reported in Table IV.

On the basis of these data it can be postulated that fractions H1 and H2b consist of two heparin chains covalently bound, while fraction H2a° contains both doublets and single chains. In keeping with this view is the close approximation

of the $\bar{M}_g$ values of H2a^0 to those of the other two fractions.

All molecular weights were calculated using a partial specific volume of 0.56 ml.g^{-1} which is somewhat higher than the values previously reported for heparin preparations (0.42 to 0.50 ml.g^{-1}; Lasker and Stivala, 1966). This discrepancy is probably due to the presence in our preparation of a protein moiety (see Table I) which accounts for about 10 per cent of the total dry weight.

Chemical and physical chemical analysis suggests that fractions H1 and H2b are similar if not identical. It was therefore decided to restrict further studies, with a view to demonstrating a covalent linkage of the polysaccharide and protein moieties, to fraction H2b since it represented the major component of bovine liver capsule and because of this high yield was more amenable to provide good sampling.

High voltage paper electrophoresis (Figure 7) showed the material to migrate to the anode as a

Figure 7

High voltage electrophoresis of the proteoheparin sample (A) compared with a commercial heparin sample (B). The run was performed on strips (35 x 58 cm.) of Whatman 3MM paper in a 1.2M-pyridine - 0.17M-acetic acid (pH 6.0) buffer at 1000V and 80mA. The chromatogram was stained with toluidine blue.

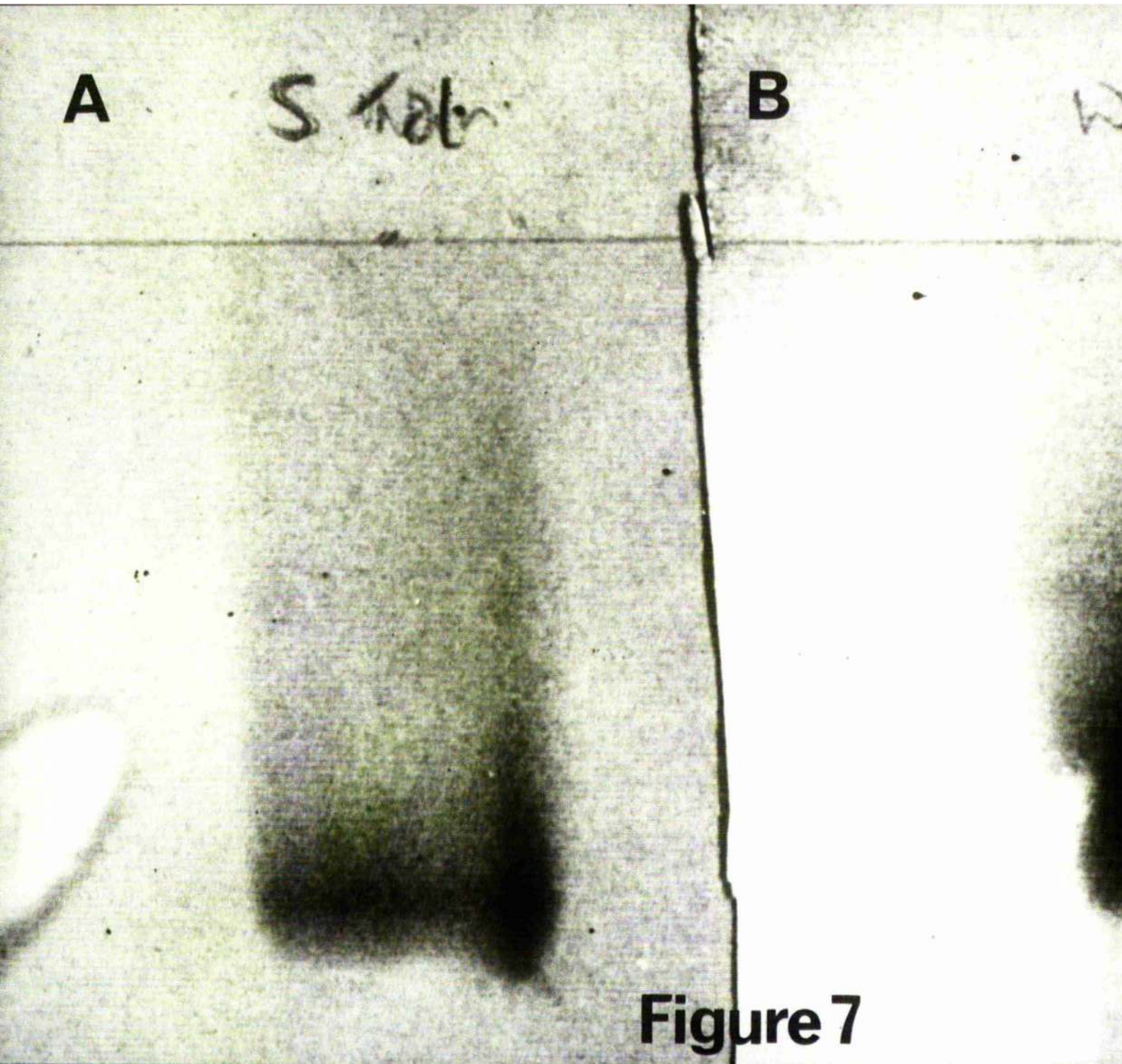


Figure 7

single component and very little heterogeneity was observed, particularly when compared with a sample of a commercial preparation. The samples were stained with toluidine blue.

Acrylamide disc gel electrophoresis (Figure 8) also showed the material to migrate as a single band which was evenly stained with a variety of dyes for protein and glycosaminoglycans.

From the electrophoresis experiments the following conclusions can be drawn:

(a) The fraction is free from contaminating material, and

(b) the protein and glycosaminoglycan moieties migrate together in spite of charge and size differences, although it is rather difficult to compare accurately the position of bands in different gels of similar characteristics which have been run under identical conditions.

The infrared spectrum of fraction H2b is shown in figure 9, and of particular importance is the absorption at 1230 cm^{-1} which corresponds

Figure 8

Disc gel electrophoresis of proteoheparin (H2b) after the gels had been stained with bromocresol green (A), amido black (B) and toluidine blue (C). All gels (7.5 per cent acrylamide) were run under identical conditions (4mA per gel) using a gel buffer solution of 377mM-Tris-HCl, pH 8.4, in conjunction with a tank buffer solution of 38mM-glycine, pH 8.2.

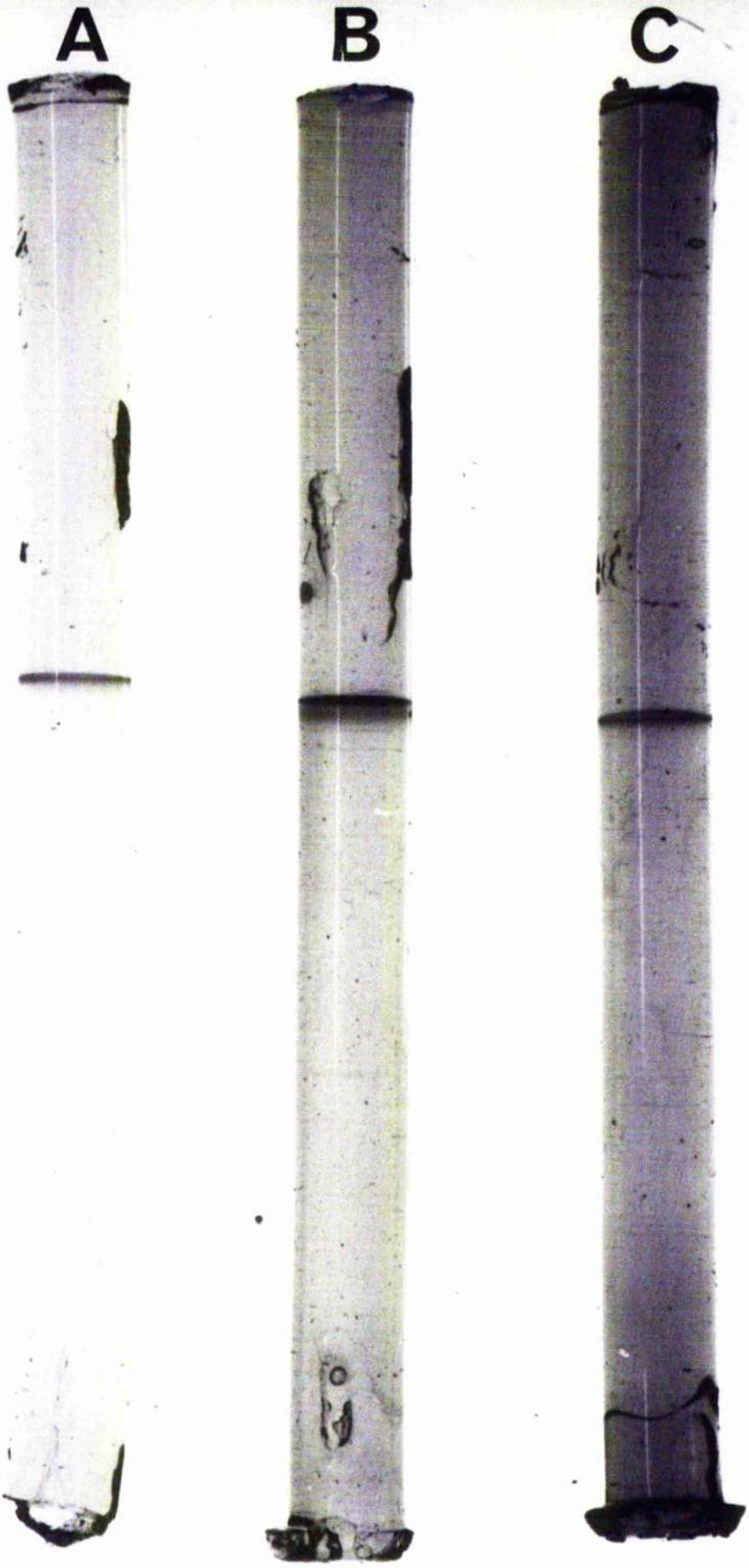
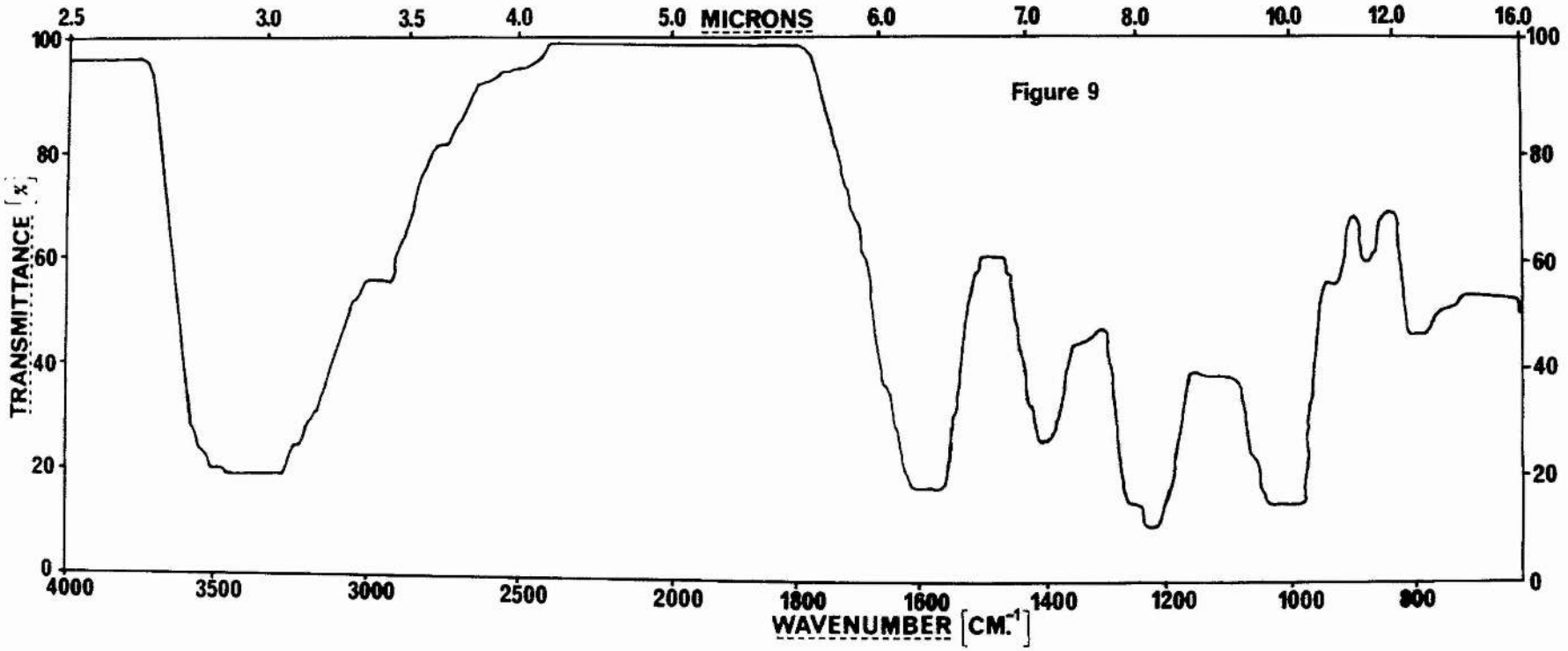


Figure 8

Figure 9

The infrared spectrum of proteoheparin (H2b)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3.5
4.0
5.0 MICRONS
6.0
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8.0
10.0
12.0
16.0
100
80
60
40
20
0
TRANSMITTANCE [%]
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
WAVENUMBER [CM.-1]

Figure 9

to the N-sulphate group on the glucosamine residue [Dietrich, 1968]. Other strong absorptions were found in the region from 700 to 1000 cm^{-1}. The band at 875 cm^{-1} can be assigned according to the findings of Dietrich to the N-sulphate glucosamine units while absorptions at 800 and 925 cm^{-1} reflect the presence of O-sulphate substituted groups. It has further been suggested that the O-sulphate group assigned at 800 cm^{-1} is associated with the hexuronic acid component [Dietrich, 1968]. Furthermore, the amount of N-sulphated glucosamine residues present in the preparation was found to be 16.3 per cent of the dry weight. However, since this macromolecular complex contains 18.4 per cent glucosamine (Table I) it is assumed that the remaining amino sugar residues are N-acetylated. This is in conformity with the observation that the region of the heparin chain in the vicinity of the potentially reducing end contains N-acetyl glucosamine residues [Lindahl, 1966; Kotoku et al., 1967]. In this respect it is important to note that biosynthetic studies on mouse mastocytoma

tumour extracts have shown that glucosamine units are probably laid down during polysaccharide synthesis in the N-acetylated form [Silbert, 1963; Helting and Lindahl, 1972].

In an attempt to establish the covalent nature of the linkage between the "protein moiety" and the heparin chains β-elimination of the carbohydrate moiety was carried out under mild alkaline conditions. However, the reaction appeared to be sluggish and only a small proportion of the chains present seemed to be affected by the reaction. Molecular weight analysis on the β-eliminated material (Table V) shows a reduction in the molecular weight averages both in the whole-cell estimations and at vanishing concentrations. The most notable decrease in molecular weight is observed at the meniscus where $\bar{M}n$ app gives a value of 9.81×10^3 daltons. This figure is in direct agreement with the chain weight value which was estimated from chemical data (Table IV) and confirms that β-elimination had occurred

TABLE V

This Table illustrates the average molecular weight values obtained after treating the proteoheparin fraction (H2b) with: (a) alkali (0.5M-NaOH), and (b) enzymically (papain and pronase). The molecular weight values recorded were obtained under identical experimental conditions to those previously reported (Table IV) and are compared with values of an untreated proteoheparin sample.

TABLE V

Sample H2b	Whole-cell		Meniscus			Column base		
	$\bar{M}_w \times 10^{-3}$	$\bar{M}_z \times 10^{-3}$	$\bar{M}_n \times 10^{-3}$	$\bar{M}_w \times 10^{-3}$	$\bar{M}_z \times 10^{-3}$	$\bar{M}_n \times 10^{-3}$	$\bar{M}_w \times 10^{-3}$	$\bar{M}_z \times 10^{-3}$
Untreated (from Table IV)	19.94 $\pm$0.14	20.77 $\pm$0.51	16.22 $\pm$0.24	18.72 $\pm$0.25	16.76 $\pm$2.44	20.20 $\pm$0.11	21.37 $\pm$0.40	28.89 $\pm$2.51
β-eliminated	14.48 $\pm$0.29	17.23 $\pm$0.47	9.81 $\pm$0.26	12.08 $\pm$0.49	14.62 $\pm$1.90	14.37 $\pm$0.11	17.42 $\pm$0.38	20.25 $\pm$2.40
Papain treated	13.37 $\pm$0.37	16.95 $\pm$0.37	8.50 $\pm$0.23	10.50 $\pm$0.66	16.04 $\pm$2.25	14.12 $\pm$0.23	18.24 $\pm$0.49	18.95 $\pm$3.30
Pronase treated	11.99 $\pm$0.44	16.88 $\pm$0.60	6.80 $\pm$0.18	7.97 $\pm$0.43	13.33 $\pm$0.86	12.56 $\pm$0.34	17.91 $\pm$0.31	24.79 $\pm$0.18

although not completely since values determined at the base of the column were found to be similar to the values reported for an untreated sample. These results show that the heparin chains are linked through an alkaline labile bond to a hydroxyamino acid(s) buried in the protein core and that the conformation of the macromolecule must bear some influence on the efficiency of the reaction. It may be considered that the xylose-linked serine residue(s) are situated at the N or C-terminal positions [Neuberger et al., 1966], however N-terminal analysis did not support this view.

Molecular weight analyses of fraction H2b which had been subjected to papain and pronase treatment support the view that in both instances the protein backbone is cleaved since all molecular weight values showed a marked decrease when compared with those of the parent preparation. In particular, the $\bar{M}n$ app value at vanishing concentration for both experiments agrees quite favourably with the

equivalent value obtained from β-elimination studies and chain weight determinations. This evidence, although not totally conclusive, confirms the presence of covalently bound polysaccharide units linked to a protein structure. However, it is worth noting that enzymic degradation does not go to completion since a proportion of high molecular weight material is still present at the base of the column after prolonged digestion. One could postulate that owing to the presence of two highly charged heparin chains in close proximity the enzymic action is sterically hindered.

The "protein moiety" was isolated after treatment of H2b with nitrous acid or heparinase.

(a) Treatment with nitrous acid at low temperature results in the conversion of N-sulphated but not of N-acetylated, hexosamine residues to anhydromannose units with concomitant cleavage of the corresponding glycosidic bonds. The "protein moiety" was isolated by ion exchange and high voltage paper electrophoresis and exhibited a

glucosamine : serine molar ratio of 0.03.

(b) Heparinase treated H2b yielded a similar glucosamine : serine molar ratio after the respective purification steps. Specificity of the enzyme requires the presence of O-sulphate and sulphamido groups in the glucosamine residues and derivatives containing free amino or N-acetyl groups are not substrates for the enzyme.

The amino acid compositions of the "protein moiety" isolated after these two procedures are shown in Table VI. On comparison with the undegraded sample (Table II), very little deviation in amino acid content is observed. Assessment of optimum hydrolysis times for glycosaminoglycan samples has shown that the recovery of serine is dependent upon the presence of hexosamine in the sample. Although on average an hydrolysis time of 24 h is taken to give optimum yields for most proteins, in the case of heparin and PP-L, maximum recoveries for some amino acids are observed after only hydrolysing the sample for 16 h. This suggests that the presence

TABLE VI

The amino acid values were corrected for hydrolytic losses (see Table II), and are expressed as residues per 1000 residues.

TABLE VI

Amino acid analysis of Fraction H2b after nitrous acid and heparinase treatment compared with an untreated sample.

Amino acid	Untreated	Nitrous acid	Heparinase
Hydroxyproline	0.0	0.0	0.0
Aspartic acid	72.7	81.5	84.4
Threonine	36.3	38.5	43.3
Serine	181.5	197.6	199.9
Glutamic acid	114.7	137.4	134.5
Proline	36.3	30.1	44.1
Glycine	224.8	255.7	272.8
Alanine	83.7	77.6	89.7
Valine	43.5	39.5	40.3
Cystine ($\frac{1}{2}$)	0.0	0.0	0.0
Methionine	0.0	0.0	0.0
Isoleucine	24.5	24.7	24.3
Leucine	71.3	46.0	45.6
Tyrosine	Trace	Trace	Trace
Phenylalanine	30.5	17.8	13.7
Hydroxylysine	0.0	0.0	0.0
Lysine	24.0	18.4	12.9
Histidine	24.0	17.8	12.9
Arginine	32.4	17.3	21.3

of the glycosaminoglycan chains bears some marked effect on the cleavage of the peptide bond.

The "protein moiety" was tested for homogeneity by two independent procedures:

(a) N-terminal analysis by the dansylation technique revealed alanine to be the only N-terminal amino acid residue present; this result was supported by the observation that the alanine content of the hydrolysate fell markedly after treatment of the protein with dansyl chloride.

(b) Acrylamide disc gel electrophoresis in which samples were stained with coumassie blue clearly demonstrated the presence of a single band (Figure 10). The sample migrated with a marker dye which suggests the presence of a small uniform molecular species.

Carbohydrate analysis of the "protein moiety" revealed the presence of neutral sugars and N-acetylhexosamines (Figure 11). The latter observation is in agreement with the finding mentioned earlier that sulphate content decreases near the

Figure 10

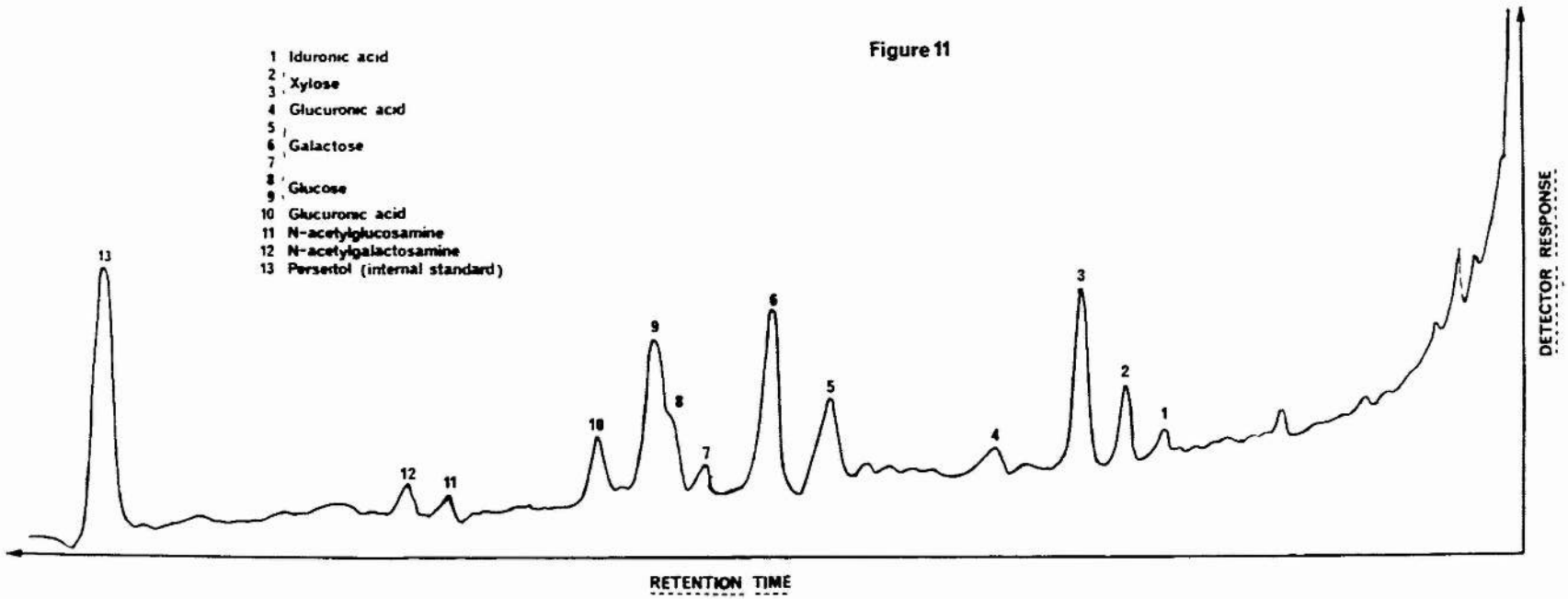
Disc gel electrophoresis run of the "protein moiety", obtained after fraction H2b had been treated with heparinase (A) and nitrous acid (B). The gels were run under identical conditions and stained using the Coumassie blue technique [Diezel et al., 1972].

Figure 10

Figure 11

Gas-liquid chromatogram of the sugars associated in the linkage of the heparin chains to the protein backbone.

Figure 11


1 Iduronic acid
2, 3 Xylose
4 Glucuronic acid
5, 6, 7 Galactose
8, 9 Glucose
10 Glucuronic acid
11 N-acetylglucosamine
12 N-acetylgalactosamine
13 Persertol (internal standard)
DETECTOR RESPONSE
RETENTION TIME

potentially reducing end of the heparin chain. However, the main feature of the gas chromatogram in figure 11 is the presence of galactose and xylose in a molar ratio of about two, which is in agreement with results recorded in Table I, and is consistent with the linkage regions of the heparin chains still being attached to the "protein moiety". Furthermore the identification from the monosaccharide profile of a high molar ratio of glucuronate to iduronate together with a glucuronate : xylose molar ratio of one indicates that these linkage regions are intact.

The chromatogram also shows the presence of N-acetylgalactosamine in addition to N-acetylglucosami The presence of the former hexosamine, which constitut 0.5 per cent of the parent complex, confirms the possibility of this unit being an integral part of the heparin chain near the reducing end. It would therefore appear that the following pentasaccharide may be present in the preparation:

$$- \begin{pmatrix} \text{Glc NAc} \\ \text{Gal NAc} \end{pmatrix} - \text{Glc UA} - \text{Gal} - \text{Gal} - \text{Xyl} - \text{Protein}$$

In order to determine the point of linkage in

the protein backbone, the "protein moiety" isolated after either nitrous acid or after heparinase treatment was subjected to alkaline conditions in the presence of sulphite ions. The reaction which was first described by Simpson *et al.* (1972) involves β-elimination of substituted seryl and threonyl residues followed by α-β nucleophilic addition of sulphite ions to the correspondin dehydroamino acids resulting in stoichiometric formation of their sulphonic acid derivatives: cysteic acid and 2-amino-3-sulphonylbutyric acid, respectively. The results are presented in Table IV. Unfortunately cystei acid and 2-amino-3-sulphonylbutyric acid are not resolved on an automatic amino acid analyser. After β-eliminatio the amino acid analysis of fraction H2b revealed a decrea in serine content (about 80 residues per 1000 residues) together with the appearance of a sulphonic acid derivati Since no loss of threonine was observed the sulphonic aci concentration was calculated as cysteic acid and a value of 74.9 residues per 1000 residues was obtained.

The electron microscopic appearance of the proteo heparin complex (H2b) is shown in figure 12. Although th particles exhibited considerable variation in size, the majority are 3nm in diameter, and are either isolated or aligned in short rows.

Figure 12

Monolayer of proteoheparin macromolecules (H2b) sprayed on to a carbon-coated grid at a concentration of 5µg per ml. The bismuth nitrate staining technique was used. Arrows (a) indicate short rows of beaded filaments composed of several 3nm particles and arrow (b) point to an isolated particle.

Figure 12

D I S C U S S I O N

DISCUSSION

Although a number of workers have claimed, [Lloyd *et al.*, 1967; Serafini-Fracassini and Durward, 1968], and in a number of cases dismissed [Lindahl, 1970; Ögren and Lindahl, 1971] the possibility of a proteoheparin complex existing in the native state, it is firmly established that the biosynthesis of the glycosaminoglycan chains occurs in the presence of an endogenous protein acceptor [Grebner *et al.*, 1966].

The existence of free heparin chains which have been reported by Lindahl (1970) to be present in bovine liver capsule may result from the proteoheparin being degraded within the mast-cell granule immediately after synthesis or, alternatively, when its physiological life-span has elapsed. In the latter case it should be possible to obtain the complex in its native state if precautions are taken to avoid enzymic degradation during isolation since it is known that heparinase(s) and trypsin-, chymotrypsin-like proteases are present in mast cells [Jaques and Cho, 1954; Benditt and Arase, 1959; Lagunoff and Benditt, 1963]. The extraction procedure developed during the course of this work was

designed to minimise the effects of these enzymes throughout all extraction and purification stages.

Infrared spectroscopy as well as chemical studies support the view that the glycosaminoglycan isolated in this work was heparin since they all revealed the presence of N-sulphated glucosamine units. The preparations were also susceptible to nitrous acid treatment and heparinase activity. Chemical evidence (see Table I) also shows that the heparin chains present in the respective preparations were intact, since all fractions were found to contain sugars known to be involved in the linkage region, in a concentration corresponding to a chain weight of about 8.0×10^3 daltons for the glycosaminoglycan. The possibility of these neutral sugars being constituents of contaminating glycosaminoglycans can be dismissed, since fractions II1 and II2b contained practically no galactosamine. Moreover even in the case of fraction II2a^0 the amount of this amino sugar was too low to account for the high concentration of xylose estimated in the sample. Furthermore chain

weights of the glycosaminoglycans estimated from both chemical and physical chemical data are in agreement with the values reported for single heparin chains from the same source [Lindahl, 1970]. It would therefore appear that free heparin chains lacking an intact linkage region [Lindahl, 1970] are not true constituents of bovine liver capsule. It seems quite probable that these degraded glycosaminoglycan chains (only one in eight were estimated to contain a xylose residue) had arisen during the course of the isolation as products of protease(s) and polysaccharidase(s) activities.

All preparations were found to contain appreciable quantities of protein ranging from 7.6 to 10.7 per cent. Direct comparison of the amino acid composition of fractions H1 and H2b was difficult owing to the low yields of the former fraction and, as a result, accurate corrections for hydrolytic losses could not be made. Nevertheless the two profiles did appear to be similar although the presence of hydroxyproline in fraction H1

indicated the possibility of collagen contaminating the preparation. Comparison of fraction II2b with II2a^{0} is probably meaningless since the latter preparation contains a relatively high proportion of galactosamine which has been interpreted as indicating the presence of dermatan sulphate as a contaminating glycosaminoglycan. Furthermore, the low molecular weight averages, together with a lower overall protein content, suggest that this preparation may be a degradation product of the parent compound.

The amino acid composition of fraction II2b, and of its protein core isolated after treating the sample with nitrous acid and heparinase are particularly interesting. Analyses were carried out on three separate preparations in order to test and correct for any contaminating material, and the respective profiles are shown in Tables VI and VII. Results reported in Table VII are expressed as the number of amino acid residues present in the "protein moiety" when the values for proline and threonine are taken as one. On this basis the "protein moiety" of the

TABLE VII

Amino acid	Untreated	Nitrous acid	Heparinase
Hydroxyproline	0.0	0.0	0.0
Aspartic acid	2.0	1.9	2.0
Threonine	1.0	0.9	1.0
Serine	5.0	4.6	4.7
Glutamic acid	3.2	3.2	3.1
Proline	1.0	0.7	1.0
Glycine	6.2	6.0	6.4
Alanine	2.3	1.8	2.1
Valine	1.2	0.9	0.9
Cystine ($\frac{1}{2}$)	0.0	0.0	0.0
Methionine	0.0	0.0	0.0
Isoleucine	0.7	0.6	0.6
Leucine	2.0	1.1	1.1
Tyrosine	0.0	0.0	0.0
Phenylalanine	0.8	0.4	0.3
Hydroxylysine	0.0	0.0	0.0
Lysine	0.7	0.4	0.3
Histidine	0.7	0.4	0.3
Arginine	0.9	0.4	0.3

The number of amino acid residues present in the protecheparin sample (H2b) before, and after nitrous acid and heparinase treatmen

undegraded preparation, which contains a single N-terminal amino acid residue, was found to comprise of a maximum of twenty-eight amino acids and consequently to have a molecular weight of 2740 daltons. Values for isoleucine, phenylalanine, lysine and histidine exhibited ratios which were somewhat lower than unity, and as a result these amino acids may represent trace contaminants which are bound electrostatically to the heparin chain, owing to their overall basic properties. On the other hand they may represent the presence at the C-terminal end of a polypeptide sequence which is particularly susceptible to proteolytic attack. The theoretical molecular weight of a compound containing such a polypeptide linked to two heparin chains would be 2.1×10^4 when molecular weights of 1.7×10^4 and 1.4×10^3 are assigned to the heparin moiety and the linkage regions respectively. This value is in agreement with the average molecular weight estimated by ultracentrifugation.

The amino acid profiles of the protein moiety

after nitrous acid and heparinase treatment of the proteoheparin sample show that the same number of residues appearsto be present for the majority of amino acids. However, the comparison of the residue composition of such preparations with that of the untreated sample clearly shows that one leucine residue is lost and a sharp reduction in phenylalanine, lysine, histidine and arginine also occurs. The fact that in both instances heparin chains have been eliminated and that the purification of the "protein moiety" by high voltage electrophoresis had yielded only a single species appears to substantiat the view that these odd amino acids may be an integral part of the polypeptide. Such a reduction in amino acid content might reflect lability of the region at the C-terminal towards acid and proteases which may contaminate the heparinase preparation.

Examination of the sugar content of the isolated protein moiety revealed the presence of two intact linkage regions for polysaccharide attachment and so it seems probable that if the methods used for

isolating this heparin-free fraction have no adverse effect on this region. While β-elimination of the parent compound was rather slow and incomplete, treatment of the "protein moiety" with alkali in the presence of sulphite ions resulted in the production of cysteic acid. Since this sulphonic acid derivative represented 37.5 per cent of the serine molar content prior to alkali treatment, the involvement of two serine residues from a possible five in the linkage of the polysaccharide chains to the polypeptide backbone appears to be confirmed.

The data so far discussed, together with the results from ultracentrifuge analysis, appear to agree with a model in which the polypeptide, containing twenty-eight amino acid residues, is covalently bound to two heparin chains through two seryl residues.

Information so far available concerning the structure of this complex does not allow postulations to be made regarding the actual location of these seryl residues involved in the linkage, but it would appear

that the protein core is buried within the macromolecular domain of the glycosaminoglycan and is consequently protected against attack from proteolytic enzymes. In fact treatment with papain and pronase was not a total success since cleavage of the protein backbone did not yield single monomeric units.

If the proposed structure of this complex is correct, then the majority of the proteoheparin species present in bovine liver capsule would have a macromolecular organisation similar to that of bovine nasal cartilage proteoglycan which contains chondroitin sulphate chains located on its protein core in a regular fashion. The overall molecular weight of this proteoglycan complex varies from 1 - 4 x 10^6 daltons. The chondroitin sulphate chains are located in pairs which are covalently bound to seryl residues separated by ten amino acids and constitute what is referred to as a doublet [Mathews, 1971]. Although there is a marked variation in these two macromolecular

complexes it is of interest to point out that proteoglycans of a molecular weight lower than 1×10^6 have been observed in porcine cartilage [Simpson and Davidson, 1972; Woodward et al., 1972], and in rabbit auricle cartilage [Serafini-Fracassini and Stimson, 1971]. Similarly it is worth noting that recent work in our laboratory has shown that bovine nasal cartilage proteoglycan represents a system of self-associating subunits in which aggregates form, probably through hydrophobic interactions of the respective protein moieties. The size of these subunits is less than 2×10^5 daltons and their fine structure is at present still under investigation.

There is evidence that some damage of the protein core may have occurred during the isolation of the sample, thus making it impossible to assess whether the 2×10^4 molecular weight species represent the native proteoglycan or degradation products of larger macromolecules. However it is worth noting that data from ultracentrifugation analysis is not in

keeping with this view, since an $\bar{M}z$ value of 2.89×10^4 daltons at the base of the column was recorded. This value may only have been obtained if macromolecular degradation had been successfully completed. It should also be considered that due to their intracellular locations, these macromolecules are probably more exposed to enzymic degradation than the corresponding intercellular glycosaminoglycans, and that it seems probable that proteolysis may start immediately after death of the animal due to the release of proteases from the lysosomes.

If the doublet structure of protoheparin is the true representation of the native complex, it would be interesting to compare its fine structure with that of extracellular proteoglycans in particular, as far as the location of the seryl residues in the protein backbone is concerned.

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