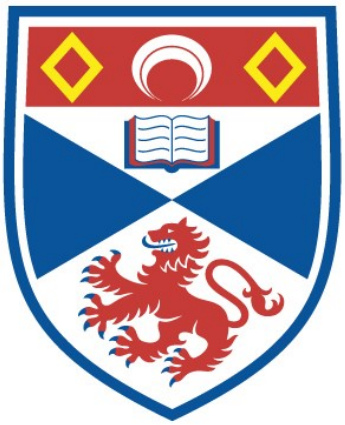
THE MACROMOLECULAR STRUCTURE OF THE HEPARIN PROTEOGLYCAN OF OX LIVER CAPSULE AND ITS ORGANISATION WITHIN THE MAST CELL GRANULE

J. J. Durward

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



1970

THE MACROMOLECULAR STRUCTURE OF THE HEPARIN PROTEOGLYCAN OF OX LIVER CAPSULE AND ITS ORGANISATION WITHIN THE MAST CELL GRANULE

by

J.J. DURWARD

A thesis presented to the University of St. Andrews for the degree of Doctor of Philosophy.

Department of Biochemistry
University of St. Andrews. 1970

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D E C L A R A T I O N

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

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

C E R T I F I C A T E

I hereby certify that J.J. DURWARD 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.

A C A D E M I C R E C O R D

I matriculated at the University of St. Andrews in October 1963, and graduated with the degree of Bachelor of Science, with first class honours, in June 1967.

From October 1967 I have been a Research Student in the Department of Biochemistry, University of St. Andrews.

A C K N O W L E D G M E N T S

I thank Dr. A. Serafini-Fracassini for his constant help and encouragement during the course of this study.

I also thank Mrs. A. Serafini-Fracassini for amino acid analyses and Dr. J.W. Smith for his advice on the preparation of sections for electron microscopy.

Thanks are also due to the Science Research Council for financial support.

C O N T E N T S.

CONTENTS (Contd.)

INTRODUCTION

CHEMISTRY OF HEPARIN

Although heparin is not strictly a structural component of connective tissue, it is closely related to heparan sulphate which is found throughout the body in such locations as cartilage and bone (Meyer et al., 1956), granulation tissue (Berenson & Dalfères, 1960), cervix uteri (Loewi & Consden, 1962) and aorta, where it is involved in the ageing process (Kaplan & Meyer, 1960).

Heparin and heparan sulphate are composed of equimolar amounts of hexosamine and hexuronic acid, when their uronic acid content is determined by the decarboxylation method (Tracey, 1948). While heparin is similar to the chondroitin sulphates in this respect, Wolfrom and coworkers (1950) demonstrated that 1-3 hexosaminide linkages could not exist in heparin since, during treatment with periodic acid, which is specific for glycol groups, all the uronic acid is destroyed. Moreover, experiments using heparin-adapted enzymes from Flavobacteria (Hoffman et al., 1957; Linker et al., 1960; Linker & Sampson, 1960) indicate that these linkages are of the 1-4 type. This was later confirmed by Foster et al., (1961) by the isolation of erythronic acid from heparin hydrolysates after periodic acid treatment.

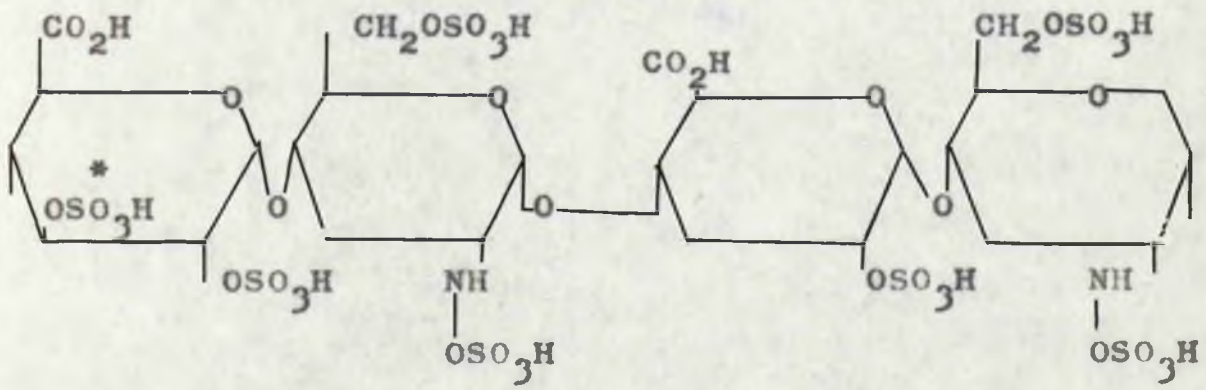
The uronide linkages in heparin cannot be of the 1-3 type, since 3-O-methylglucose has been isolated from hydro-

lysates of methylated heparin (Nominé, Burcout & Bertin, 1961). Furthermore, oligosaccharides obtained after partial hydrolysis of heparin do not show the shift in absorption maximum from 540 to 510 mu in the Elson-Morgan reaction which is characteristic of 3-O-substituted hexosamines (Danishefsky, Eiber & Langholtz, 1960; Cifonelli & Dorfman, 1961). The majority of these linkages appear to be of the 1-4 type since a disaccharide has been isolated from partial hydrolysates of desulphated carboxyl-reduced heparin, which gives half of the theoretical yield in the Elson-Morgan reaction (Wolfrom, Vercelloti & Horton, 1962). The N-acetylated derivative, on the other hand, gives no colour in the Morgan-Elson reaction (Aminoff et al., 1952). This behaviour is characteristic of 4-substituted hexosamines. Although 1-4 linkages predominate in heparin, a small proportion of 1-6 type has been demonstrated in some preparations (Danishefsky, Eiber & Williams, 1963).

The amino groups of heparin are sulphated, unlike the other glycosaminoglycans which have N-acetyl groups. Most heparin preparations contain an average sulphate content of five groups per tetrasaccharide (Muir, 1964), although their precise location has yet to be unequivocally established. It has even been suggested that these groups may not be covalently attached to the heparin chain (Helbert & Marini, 1964). This hypothesis now appears unlikely (Stivala et al., 1967). This problem is made even more complex due to the variation in

sulphur content of different preparations, and possible species differences (Walton, Ricketts & Bangham, 1966). Foster and coworkers (1961), however, have demonstrated that the majority of the sulphate groups occur at the C_2 position of the hexuronic acid moiety, although a smaller proportion has been detected at the C_3 position (Durant, Hendrickson & Montgomery, 1962). The remainder of the sulphate groups appear to be located at the C_6 position of the hexosamine (Muir, 1964).

At this time, therefore, a structure for the heparin molecule, based on a tetrasaccharide unit, was beginning to emerge, as shown below:-



* minor component.

In this model heparin appears to be basically similar to the other mucopolysaccharides, save in its N-sulphation and variable degree of ester sulphation.

In the light of subsequent research, however, this model has received a sharp set-back. The uronic acid moiety of heparin, when determined by the cabazole method (Dische,

1947; Bitter & Muir, 1962), appears to produce a colour yield appreciably higher than that obtained from other mucopolysaccharides. This anomaly can be explained by either the presence of another hexuronic acid in addition to glucuronic acid, or by some property of heparin which creates an artifact. It has been suggested that, in fact, this phenomenon is due to the N-sulphate groups (Linker et al., 1958), but more recent research, using de-N-sulphated heparin does not support this finding (Danishefsky et al., 1962). Cifonelli & Dorfman (1962) first reported the presence of iduronic acid in heparin, and this component has received a great deal of attention in recent years. Iduronic acid has been detected in the degradation products of heparin treated with a Flavobacterium Heparinum enzyme (Dietrich, 1968), and an iduronic acid to glucuronic acid ratio of 1:2.6 has been reported in heparin analysed by gas liquid chromatography (Radhakrishnamurthy et al., 1968a). Wolfrom and coworkers (1969) have isolated and crystallised iduronic acid from chemically degraded heparin and confirmed its identity by various chemical tests. These workers have also reported an iduronic acid content of 32%, and suggest that previous degradation procedures have been insufficiently mild to allow its isolation. Furthermore, on the basis of a proton magnetic resonance study, Perlin et al., (1968) have suggested that the heparin molecule is composed of a repeating trisaccharide of iduronic acid, glucuronic acid and gluco-

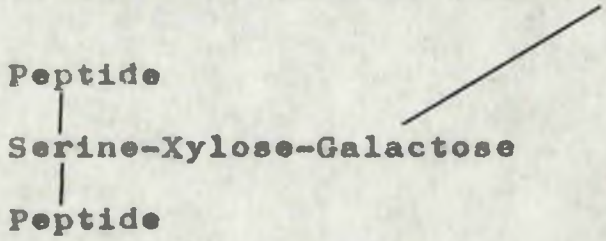
samine in equimolar concentrations.

In contrast to the widely held belief that the glucosamine content of heparin is exclusively N-sulphated. Lindahl (1966b) has demonstrated that the amino sugars in the heparin-protein linkage region are N-acetylated in most cases, and gas liquid chromatography indicates that around 20% of the total hexosamine molecules are N-acetylated (Radhakrishnamurthy et al., 1968b), while a galactosamine content of 5.6% of the total hexosamine has also been demonstrated (Radhakrishnamurthy et al., 1968a).

The precise location of the ester sulphate groups of heparin remains confused. Dietrich (1968) has identified glucosamine-2,6-disulphate and glucuronic acid-3-sulphate in the digestion products of heparin treated with a heparinase from Flavobacterium Heparinum. The localisation of ester sulphate at the C_6 position of the hexosamine has recently been confirmed by chemical techniques (Wolfrom et al., 1969; Danishefsky et al., 1969), but Danishefsky and coworkers (1969) have reported the isolation of glucosamine with sulphate groups in the C_3 and C_6 positions and C_2-sulphated glucuronic acid.

In recent years a considerable amount of research has been performed on the heparin-protein linkage region. Lindahl et al., (1965) from their study of crude commercial heparin preparations, isolated mainly from pig tissues and containing residual peptides, reported that serine was the predominant amino acid component. After chromatographic

analysis of hydrolysates of these heparin samples, two carbohydrate-serine compounds were identified, namely xylosylserine and galactosylxylosylserine (Lindahl & Rodén, 1965) and, on the basis of this result, the following structure for the heparin-protein linkage region was proposed.



Lindahl (1966a) isolated O-β-D xylopyranosyl-L-serine and 4-O-β-D-galactopyranosyl-O-β-D-xylopyranosyl-L-serine, thus identifying the configuration of the sugars. Further research (Lindahl, 1966b) indicated that the amino-sugars are N-acetylated, and two of these molecules are involved either in or near the linkage region. Moreover, all molecules in this area are devoid of any covalent sulphate indicating incomplete sulphation. In addition, iduronic acid has been detected in some of the carbohydrate-serine compounds isolated after hydrolysis.

More recently, Lindahl (1968) has demonstrated that the major disaccharide component of the linkage region is glucuronic acid 1-4 glucosamine, that is the same basic unit as that suggested by Danishefsky & Steiner (1965) for the heparin molecule as a whole, except that the amino groups are N-acetylated. Furthermore, the previous model was revised to contain two glucuronic acid residues next to the terminal

galactose molecule.

It is evident, therefore, that, although the area near to the linkage to protein has been clarified, much confusion remains concerning the structure of the remainder of the heparin molecule. The precise location of its iduronic acid moiety has yet to be conclusively identified, and sulphate groups have been detected at almost every available hydroxyl group. Furthermore, a small galactosamine component is normally found during heparin analysis, but no location has yet been postulated for this compound.

Undoubtedly much of the difficulty experienced in the determination of the structure of heparin is caused by the reliance on commercial or laboratory preparations isolated using alkali, proteolytic enzymes or by autolysis and these methods would be expected to cause damage to the material. Species differences must also create problems, and it must be remembered that heparin is an intracellular glycosaminoglycan so that many molecular species may be present, representing various stages in the biosynthesis of the molecule. These problems are reflected in the wide variation in molecular weight and biological activity of different preparations.

PHYSICAL CHEMISTRY OF HEPARIN.

The blood anticoagulant activity of heparin has been generally accepted for some time, and the majority of physico-chemical investigations have been concerned with this property of the glycosaminoglycan. This type of study has been complicated by the extreme heterogeniety of the commercial preparations normally employed, but some studies on fractionated heparins demonstrate some interesting physical parameters of the heparin molecule itself.

Reported values for the molecular weight of heparin exhibit wide variations. Light scattering experiments give molecular weights between 10000 and 19700 (Barlow, Sanderson & McNeill, 1961; Ascoli, Botre & Liquori, 1961), while values between 4000 and 17000 have been reported for the sedimentation equilibrium method (Laurent, 1961; Lasker & Stivala, 1966; Braswell, 1968).

Studies on fractionated bovine heparin (Lasker & Stivala, 1966) reveal that a commercial preparation can be separated into four fractions on ECTEOLA, with molecular weights ranging from 8460-15680, but when the same preparation is subjected to fractional alcohol precipitation, twelve fractions are obtained with molecular weights between 4000 and 13970, the molecular weights in both cases being measured by equilibrium sedimentation. Biological activity assays on these fractions showed that the higher the molecular weight, the higher the biological activity. Viscosity measurements

were also performed on the fractions, and the data obtained, when correlated with the molecular weights, indicated that the heparin molecule exhibits considerable flexibility.

Further experiments using bovine heparin fractionated using ethanol and dioxane as precipitating agents (Liberti & Stivala, 1967) produced fractions varying in molecular weight between 4500 and 15500. It was reported that biological activity varied with molecular weight in fractions up to 10000, but no appreciable difference was observed with fractions exhibiting molecular weights in excess of that figure. Further viscometric data suggested that the heparin molecule is fully extended in water, and consists of a single unbranched linear chain. The results of viscometry carried out in salt solutions confirmed earlier results (Lasker & Stivala, 1966) that the heparin molecule exhibits considerable flexibility.

Low angle X-ray scattering studies on a fraction precipitated in an ethanol-dioxane system (Stivala et al., 1968) give a molecular weight of 12900, which is in good agreement with molecular weights of 12600 and 12500 for the same fraction when measured by sedimentation equilibrium and viscosity (Lasker & Stivala, 1966). A persistence length of 21.1Å and a radius of gyration of 35.9Å were also determined, while the molecule was reported to behave in water as a Gaussian coil with a length of 237Å to 245Å.

Braswell (1968) has reported heparin fractions varying in molecular weight between 8180 and 17000. Furthermore,

the author suggests that the values reported by Lasker & Stivala (1966) may be considerably low due to the omission of a correction factor in their calculations. During his experiments Braswell found no correlation between molecular weight and biological activity.

During graded self hydrolysis of heparin (Stivala et al., 1967) the loss in anticoagulant activity is accompanied by a corresponding reduction in sedimentation coefficient, intrinsic viscosity in water and a decrease in the axial ratios a/b, if a prolate ellipsoid molecule is assumed. The viscosity in 0.5M-NaCl, however, is not affected, indicating that the decrease in molecular weight is caused by desulphation and not depolymerisation. Copper ion binding studies (Stivala & Liberti, 1967) revealed an increased uptake with increasing pH and biological activity, suggesting that the anticoagulant activity of heparin is dependent on the degree of dissociation of ionisable groups, including carboxyl groups, and its molecular size and shape. These results were essentially confirmed in similar experiments employing mild acid hydrolysis (Braswell, 1968).

Gauthier et al., (1969), however, have reported that at a certain point during mild acid hydrolysis at 25°C, 96% of the dye binding capacity of heparin is retained, whilst its biological activity is reduced by 70%. Since in these experiments only 2% of the sulphate groups account for

approximately 70% of the anticoagulant activity, these authors suggest the possibility of key bonds within the heparin molecule.

The vast distribution of molecular weights observed in all of the above investigations indicates that a considerable degree of degradation is occurring during the extraction of heparin. If degradation of this type, which appears to be causing a certain amount of depolymerisation, is taking place, then it is equally likely that many changes in chemical composition and physico-chemical parameters may also occur. Thus it is important that these data, valuable as they are, are treated with a degree of caution since they may apply to a compound very different from heparin in its native state.

THE PROTEIN COMPLEX OF HEPARIN.

Although the glycosaminoglycan-protein complex containing chondroitin 4-sulphate has been extensively characterized (Schatton & Schubert, 1954; Mathews & Lozaityte, 1958) and other complexes having chondroitin 6-sulphate (Buddecke & Schubert, 1961; Mathews, 1962) and hyaluronic acid (Ogsten & Sherman, 1958; Pigman, Rizvi & Holley, 1961; Sandson & Hammerman, 1962) as the polysaccharide moieties have been studied, the situation with regard to native heparin-protein complexes has yet to be clarified.

Perhaps the most significant previous attempt to isolate the native heparin-protein complex is that of Julén, Snellman & Sylvén (1950). In the supernatant of a phosphate-buffer extract of ox liver capsule, after centrifugation at 60000g, protein and lipid, in addition to heparin, were detected. From these findings Julén et al., (1950) concluded that the native heparin-protein complex exists in a lipoprotein form. However, the protein component was dissociated from the complex and precipitated by saturation with phosphate ions (Snellman, Sylvén & Julén, 1951), indicating the absence of covalent linkages. It is therefore considered, in agreement with Korn (1959), that this complex is a methodological artifact, due to the established affinity of heparin for lipoproteins (Bernfield, Donahue & Berkowitz, 1957).

Green, Day & Roberts (1961) reported the presence of

ninhydrin-sensitive spots in paper chromatograms of heparin hydrolysates and postulated the presence of amino acids covalently bound to carbohydrate. These results were confirmed by Lindahl et al., (1965), who demonstrated the presence of serine-rich peptides bound to heparin in preparations isolated using either proteolytic enzymes or alkali. Lindahl & Rodén (1965) also proved that the two moieties are covalently bound through a xylosylserine linkage, and suggested the existence of a native heparin-protein complex. Korn (1959), however, found no evidence for such covalent complexes in his study of heparin isolated from mast cell tumours. Lagunoff (1965), on the other hand, isolated a very small amount of a heparin-protein complex, and suggested that the heparin and protein were covalently bound. Furthermore, the existence of a heparin-protein complex in the matrix of the mast cell granules has been proposed on the evidence that a fraction extracted by potassium chloride from isolated organelles contained both heparin and protein (Lloyd, Bloom & Balazs, 1967).

MAST CELLS.

Mast cells were first recognized and described by von Recklinghausen (1863) in unstained specimens of frog mesentery. Furthermore, Waldeyer (1875) reported that in connective tissue, in addition to fibroblasts, granular cells exhibiting an "embryonal" appearance were also present. The name "Mast Cell" was first used by Ehrlich (1879), who reported that these cells could be distinguished by their metachromatic granules which he believed, at that time, to be the product of overfeeding.

In 1937, Jorpes et al. demonstrated that heparin, isolated from dog liver, stains metachromatically with toluidine blue and, in addition, that there was a good correlation between the heparin content and the mast cell count of various tissues. It had been previously reported (Biedl & Kraus, 1909) that, during peptone shock in dogs, the blood clotting time was considerably increased. Wilander (1939) observed that the hepatic mast cells of the dog also discharge their granules during peptone shock, while Jacques & Waters (1941) found that this was accompanied by the release of heparin from the liver.

Riley & West (1953) showed that the histamine content of various tissues is related to their mast cell count, and Benditt, Bader & Lam (1955) demonstrated that during anaphylactic inflammation, the discharge of the mast granules parallels the reduction in tissue histamine. Benditt and

coworkers (1955) also demonstrated the presence of 5-hydroxytryptamine in mast cells.

The localization of heparin, histamine and 5-hydroxytryptamine within the mast cell granule was established by Hagen, Barrnet & Lee (1959) using electron microscopical investigations together with ultracentrifugation, biochemical and biological assays. The concentration of these components in an average rat mast cell (expressed as micromicrograms per mast cell) have been reported to be in the range: heparin 30-40, histamine 12-31 and 5-hydroxytryptamine 0.9-1.3 (Benditt et al., 1955; Benditt, Arase & Roeper, 1956; Moran, Uvnäs & Westerholm, 1962; Humphrey, Austen & Rapp, 1963.).

In addition to these major components many enzymes have been demonstrated to be present in the mast cell granules. The most studied enzymes are the chymotrypsin-like and trypsin-like proteases. A chymotrypsin-like enzyme has been isolated from rat peritoneal mast cells (Benditt & Arase, 1959; Lagunoff & Benditt, 1963), rat thyroid (Pastan & Almqvist, 1966) and dog mastocytomas (Lagunoff & Benditt, 1963; Auditore, Ende & Katayama, 1963; Katayama & Ende, 1965) and the trypsin-like enzyme has been isolated from human spleen (Ende & Auditore, 1961) and several dog mastocytomas (Lagunoff & Benditt, 1963; Katayama & Ende, 1965). Conclusive evidence for the granule localization of the chymotrypsin-like enzyme has been provided by analyses of the

distribution of the enzyme in subcellular fractions of sonically disrupted mast cells (Lagunoff & Benditt, 1963; Lagunoff et al., 1964). It has been calculated to occupy 10% of the dry weight of the granule (Daržynkiewicz & Bernard, 1967; Lagunoff, 1968), and has a pH optimum of 8.0 and a molecular weight of 23000 (Pastan and Almqvist, 1966). The localization of this enzyme within the mast cell granule has been studied in preparations of rat peritoneal mast cells (Lagunoff, 1965). It appears to be bound, in the fully active form, to the heparin-protein matrix.

Other enzymes known to be present in mast cell granules include hydroxylases (Jordon & Hartman, 1962; Levine et al., 1964), B-glucuronidase (Lutzner, 1964), succinic dehydrogenase (Glick & Pothapragada, 1961), phosphatidase (Keller, 1962; Anggård et al., 1963) and an enzyme which oxidised cyst(e)ine to cysteic acid (Wheldrake & Pasternak, 1968).

The electron microscopic appearance of mast cells does not differ essentially from other cells, except in the possession of specific granules (Selye, 1964). The typical mast cell has a mean diameter of approximately 15 u, and contains a single, sometimes indented nucleus, a poorly developed endoplasmic reticulum and Golgi apparatus. It contains relatively few mitochondria which are often positioned adjacent to the nucleus. The nuclear and cell membranes appear to be double and the cell surface frequently exhibits villosities.

The majority of mast cell granules are very electron dense and exhibit a homogeneous granular appearance in the electron microscope (Thiéry, 1963; Bloom & Haegermark, 1965; Weinstock & Albright, 1967; Kobayasi et al., 1968). The size of the granules varies between 0.4 and 1.2 u (Bloom & Haegermark, 1965) and in some cases two types have been identified; a simple granule with a homogeneous granular appearance and a compound granule containing several areas of different appearance (Weinstock & Albright, 1967). In certain granules, mainly from human or guinea pig specimens, elongated cylindrical or scroll-like lamellae have been observed (Stockenius, 1956; Weinstock & Albright, 1967; Brinkman, 1968; Kobayasi et al., 1968).

In some granules an apparently crystalline matrix has been reported. Fedorko & Hirsch (1965), from their electron microscopic study of the mast cells of human skin, reported the presence of a honeycomb pattern in some granules, with a spacing of 120Å between the parallel dense lines forming this substructure. Weinstock & Albright (1967) observed a similar substructure in human gingivial mast cell granules. These authors described the substructure as a highly ordered array of parallel, dense and less dense lines with a periodicity of 70Å, or, if sectioned at 90° to this plane, a hexagonal latticed network with a centre-to-centre spacing of 70Å. A similar type of substructure has also been observed in the granules of mast cells in patients suffering

from uticaria pigmentosa (Kobayasi et al., 1968). This type of ultrastructure, however, has yet to be demonstrated in rat peritoneal mast cell granules.

Bloom & Haegermark (1965) during their studies of the substructure of rat peritoneal mast cell granules following induced histamine release, observed some granules, in untreated specimens, in which the electron density was considerably reduced, revealing a substructure composed of an extremely fine filamentous meshwork in which electron dense particles of 75-100Å were visualized. The number of granules of this type increased in frequency after histamine release. Granules exhibiting this type of structure, although not reported, can be observed in electron micrographs of mast cells from human bronchus (Brinkman, 1968), human skin (Kobayasi et al., 1968) and human gingiva (Weinstock & Albright, 1967). Examination of these granules by light microscopy reveals a more intense metachromasia, implying that the filamentous network is composed of heparin (Bloom & Haegermark, 1965). This conclusion is supported by the observation of Archer (1961) that very little heparin is released from the mast cell granules during histamine release. Lloyd et al., (1967) also observed a network of fibres within mast cell granules. Extraction of the mast cells with 2M-KCl caused the dissolution of this network, and a heparin-protein complex was identified in this extract (Lloyd, Bloom & Balazs, 1967).

MATERIALS AND METHODS.

ISOLATION AND PURIFICATION OF THE HEPARIN PROTEOGLYCAN OF OX LIVER CAPSULE.

The capsule, stripped free from liver tissue, was defatted in acetone, dried and powdered. Portions, 20g each, of this material were extracted in a blender with 400ml of a 1% (w/v) solution of cetylpyridinium chloride in 2M-KCl for 1h. After centrifugation at 38000g av. for 1h at 4$0C^{o}$, the clear supernatant was diluted to give a final concentration of 0.9M-KCl. The fine precipitate that gradually formed at room temperature during 4h was collected by filtration on a cellulose filter pad, the filtrate being discarded. The pad was eluted with 100ml of 2M-KCl, at 4$0C^{o}$, and the cetyl-pyridinium ions dissociated from the complex and removed by shaking with 3 vol. of chloroform-pentan-1-ol (5:4 v/v) (Scott, 1960) at room temperature. The aqueous phase, when the cetylpyridinium chloride had been extensively eliminated, as verified by the disappearance of its characteristic E_{260}, was dialysed against glass-distilled water for 48h at 4^{o}C. After stepwise addition of 4 vol. of ethanol at room temper-ature, the solution was centrifuged at 38000g av. for 1h. Then 1 vol. of a saturated solution of potassium acetate in ethanol was slowly added to the supernatant. The white precipitate that then formed was collected by centrifugation, washed in acetone and dried. The material was redissolved in water and the ethanol fractionation repeated twice. The

final dry powder was stored in a desiccator over P_2O_5 until required for analysis.

BLOOD ANTICOAGULANT ASSAY.

The blood anticoagulant activity of the preparation was determined by the method of Jacques (1943) as modified by Sharp, Excell, Salzman & Thorup (1961). This method involves the titration of the heparin preparation with protamine and the comparison of its activity with that of a standard preparation (Boots Ltd., crystalline, cresol-free).

ASH AND MOISTURE CONTENT.

The ash and moisture content of the preparation was determined by the method of Eastoe & Courts (1963).

AMINO ACID ANALYSIS.

Samples of the preparation were hydrolysed for amino acid analysis in constant-boiling HCl (2ml/mg of material) under nitrogen, in sealed tubes at 110°C for 24, 36 and 72h. Excess of acid was removed from the hydrolysates in a rotary film evaporator at 30°C. Owing to the presence of cyst(e)ine, separate portions of the material were oxidised with performic acid (Schram, Moore & Bigwood, 1954) before hydrolysis. The amino acid analyses were carried out in triplicate with a Technicon AutoAnalyzer. The reproducibility of the results was about ± 1.5%. Superimposition of valine and glucosamine peaks was prevented by using an initial buffer of pH 2.75 and by replacing 10 and 7ml of buffer in the first and second

chambers of the Autograd with methanol.

HEXOSAMINE ANALYSIS.

The hydrolysis for hexosamine analysis was carried out in 4M-HCl (2ml/mg of material) at 100°C for 10h (Wolfrom, Weisblat, Karabinos, McNeely & McLean, 1943) under nitrogen in sealed tubes. Excess of acid was neutralized and total hexosamine was determined by the method of Cessi & Piliego (1960).

This procedure involves the distillation of the volatile fraction of chromogens formed by heating amino sugars with acetylacetone in an alkaline medium, thereby preventing the interference of amino acids during colour production. Ogsten (1964), however, pointed out that the estimation of hexosamines in complex polysaccharides may give low results because other substances present may be converted, during hydrolysis, into products that subsequently decrease the yield of chromogen formed in the reaction with acetylacetone. To check this source of error, Serafini-Fracassini, Peters & Floreani (1967) hydrolysed samples of bovine haemoglobin, ossein gelatin and serum albumin together with known quantities of hexosamine under the conditions specified for hexosamine determination. The results of this investigation showed that haemoglobin did not interfere even in 1000 fold excess, gelatin lowered the yield if in 200 fold excess, and albumin, in the range 200-2000 fold excess, had the opposite

effect. None of the proteins tested caused any significant interference in the range used in the present investigation.

The concentrations of glucosamine and galactosamine were determined after chromatographic separation (Partridge & Elsden, 1961).

In all cases the values were corrected for losses during hydrolyses.

HEXURONIC ACID ASSAY.

Quantitative determination of hexuronic acid was performed by the method of Bitter & Muir (1962). This method is a modification of the carbazole method of Dische (1947). The use of borate in sulphuric acid is claimed to increase sensitivity, increase the stability of the colour, which is developed immediately, and give greater reproducibility due to reduced interference by chloride ions and oxidants.

XYLOSE ASSAY.

Xylose was determined by the anthrone method of Tsiganos & Muir (1966). This method gives good reproducibility and, due to differences in reactivity, xylose can be determined in a mixture of pentoses. Furthermore, interference due to hexoses, hexuronic acids, hexosamine and protein is negligible.

SULPHUR ANALYSIS.

The method used for sulphate estimation was that described by Giellman & Tölg (1960). The principle employed is the reduction of sulphate to hydrogen sulphide which is

distilled, in a hydrogen atmosphere, into 2M-NaOH producing sodium sulphide. The sodium sulphide formed is then titrated with cadmium solution, using dithizone as indicator.

IDENTIFICATION OF NEUTRAL SUGARS.

Samples of the complex were hydrolysed in 2M-HCl (2ml/mg of material) at 100°C for 8h. The identity of the neutral sugars present in the hydrolysate was determined by thin layer chromatography on kieselguhr G by the method of Stahl & Kaltenbach (1961).

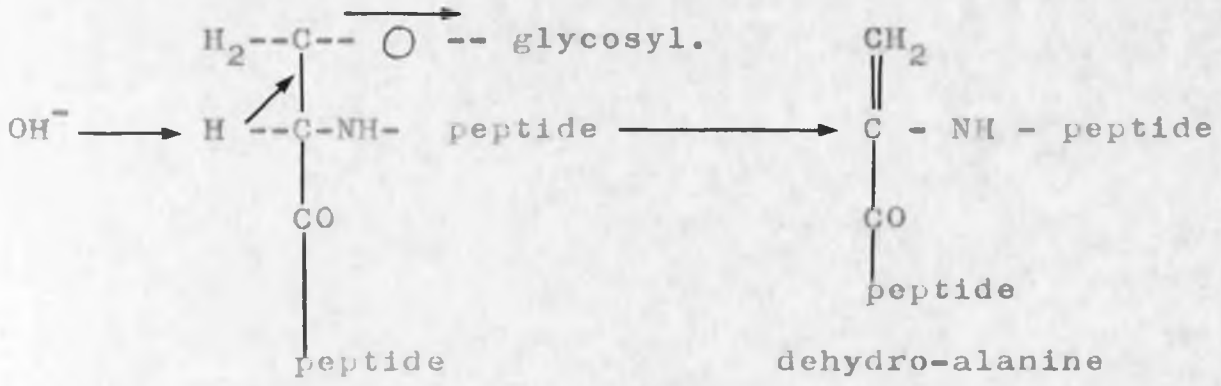
The solvent was composed of 65ml ethyl acetate and 25ml of a mixture of isopropanol and distilled water (2:1, by volume). The plates were developed in a Desaga S-chamber (Desaga Co., Heidelberg, Germany) and stained with anisaldehyde in sulphuric acid.

ALKALI-INDUCED β-CARBONYL ELIMINATION.

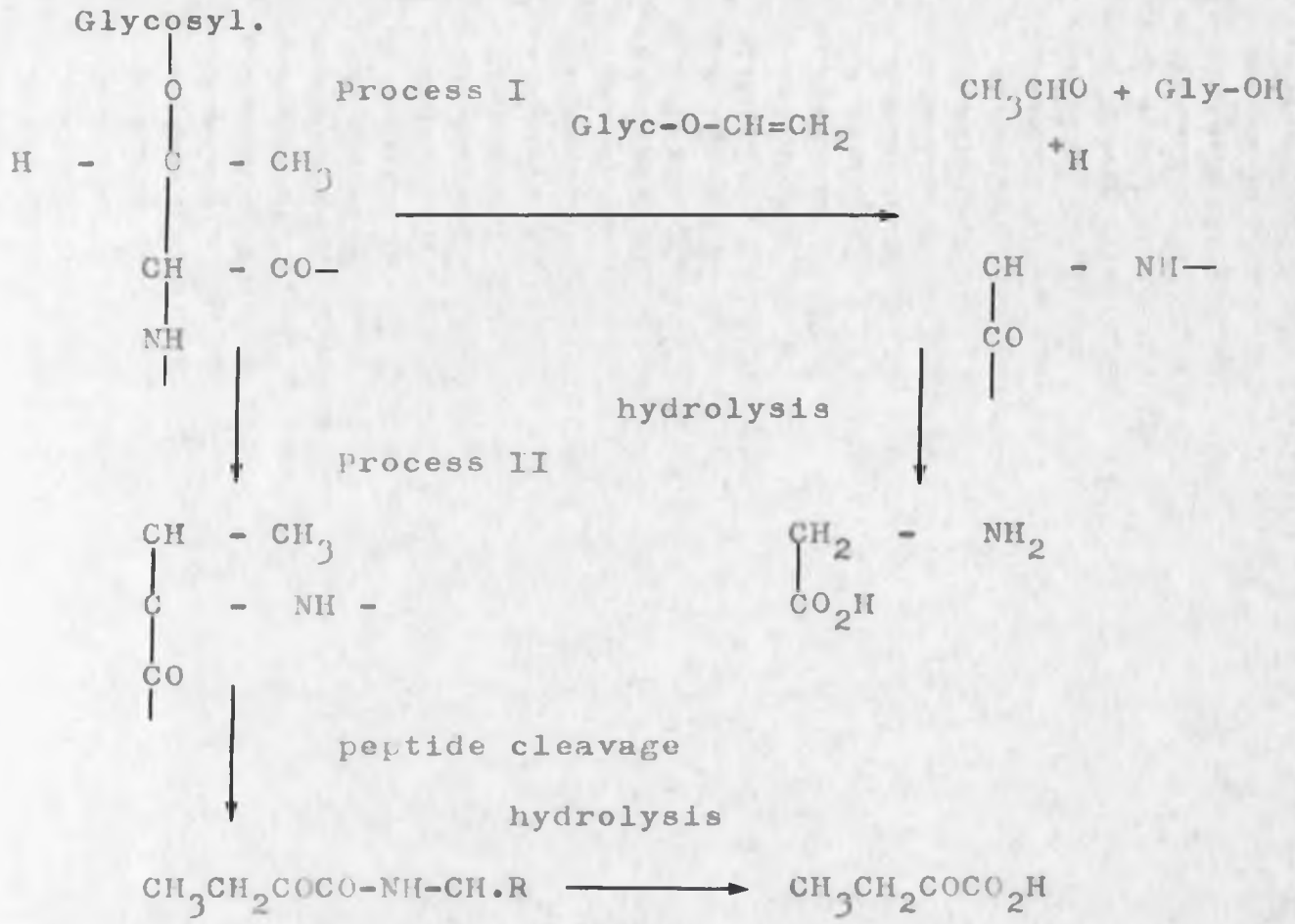
Among the methods available for demonstrating the presence of O-glycosidic linkages to the hydroxyl groups of serine and threonine residues in proteins, β-elimination is probably the most widely employed. If a glycoprotein contains such a linkage, then prolonged treatment with dilute alkali, followed by amino acid analysis reveals a decreased concentration in the particular amino acid involved.

Neuberger, Gottschalk & Marshall (1966) have reported a mechanism for this reaction, when serine is involved in O-glycosidic linkage. The masking of the amino and carbonyl

groups of the amino acid, in proteins, increases the acidity of the α-hydrogen atom, thereby rendering it susceptible to attack by alkali, as follows:-



If this reaction is carried out in the presence of Adams catalyst, under hydrogen, then amino acid analysis reveals a loss in serine concentration and a corresponding increase in alanine due to the reduction of dehydroalanine. β-elimination of O-substituted threonine occurs by a similar mechanism, but Adams (1965) has proposed that threonine follows two separate pathways:-



$CH_3CH_2COCO\text{-}NH\text{-}CH.R \longrightarrow CH_3CH_2COCO_2H$

After alkaline treatment, therefore, one would expect the amino acid analysis to reveal a reduction in threonine content and an increase in glycine content. If this reaction is carried out in the presence of Adams catalyst, under hydrogen, the intermediate formed in process II would be reduced, yielding α-aminobutyric acid. Tanaka et al., (1964) have reported, however, that only 15% of the threonine lost was recovered as α-aminobutyric acid in material treated in this way, and Anderson et al., (1964) could account for only 35% of the threonine lost as α-aminobutyric acid. It would appear, therefore, that process I is also operative when hydrogenation is applied, and, in the hydrolysate of material

treated with dilute alkali, a reduced threonine content, an increased glycine content and a small amount of α aminobutyric acid should be detected.

By subjecting a glycoprotein to alkali-induced β-elimination, both with and without hydrogenation, followed by amino acid analysis, it is possible not only to demonstrate the presence of an O-glycosidic linkage, but also to identify the amino acid involved.

A 40mg sample of the material was dissolved in 10ml of 0.5 M-NaOH and stirred, under nitrogen, for 24h. The formation of the unsaturated derivative(s) of the hydroxy-amino acid(s) involved in the heparin proteoglycan linkage region was followed, by recording the change in absorbancy at 241 mu in a Beckman D.B. spectrophotometer (Riley, Turnbull & Wilson, 1957). A sample of bovine chondromucoprotein was subjected to identical treatment for comparison. A 5ml portion of this solution was then acidified to give a final concentration of 6M-HCl and hydrolysed for 24h in sealed tubes, under nitrogen, at 110°C before amino acid analysis. The remainder was neutralised and used for chromatography on AE cellulose (see below).

A separate 20mg sample was hydrogenated by the method of Anderson, Hoffman & Meyer (1965) with 20mg of Adams catalyst (Adams, Voorhees & Shiner, 1941) in 5ml of 0.5 M-NaOH for 24h at room temperature. The catalyst was removed by filtration and washed several times with small amounts of

conc. HCl. The washings were added to the initial filtrate and, after adjustment of the HCl concentration to 6M, 30ml of constant-boiling HCl was added. The hydrolysis for amino acid analysis was carried out under reflux for 24h. The presence of α-aminobutyric acid in the above hydrolysate was detected by two dimensional thin layer chromatography on silica gel G by the method of Fahmy, Neiderweisser, Pataki & Bremner (1961) using chloroform:methanol:17% ammonium hydroxide (2:2:1, by volume) in the first direction and phenol:water (3:1, by weight) containing 20mg sodium cyanide per 100g of mixture in the second. The chromatograms were developed in a Desaga S-chamber and stained with ninhydrin.

CHROMATOGRAPHY ON A.E. CELLULOSE

The adsorbent, 1m-equiv. of cationic groups per g (Whatman, W. and R. Balston, Ltd., Maidstone, Kent), after repeated washing with 0.5M-HCl and 0.5M-NaOH, was suspended in 0.1M-NaCl, degassed and poured into a 1cm glass column to a height of 30cm. Water was passed through the column and the effluent tested with silver nitrate for NaCl content. When a chloride-free effluent had been attained, the flow rate was adjusted to 12ml/h. After application of the sample, the column was washed with water for 1h. Stepwise elution was carried out with 50ml volumes of different NaCl concentrations in 0.01M-HCl; 0.25M increments in NaCl concentrations were used. Fractions, 2ml each, were collected and tested for polysaccharide content with a 1%

(w/v) solution of cetylpyridinium chloride (Scott, 1960). Chondroitin sulphate (Lot no. 15B-0010, Sigma Chemical Co., St. Louis, Mo., U.S.A.) and heparin (Lot no. 136651, Wilson Laboratories, Chicago, Ill., U.S.A.) were used as standards for the determination of their relative retention volumes.

COLUMN CHROMATOGRAPHY ON AGAROSE GEL

A column of agarose consists of two phases; a gel phase of agarose granules and a liquid phase. If the volume of the gel phase is Vx, the volume of the liquid phase is Vo and the total bed volume is Vt, then $Vt = Vo + Vx$. Now if a substance, whose molecular weight is such that its molecules cannot enter the gel, is applied to the column, then the molecules will permeate only the liquid phase (Vo) and will be eluted immediately after the void volume (Ve). If, however, the molecular size is such that a certain volume of the gel particles is accessible to them, then there will tend to be an equilibrium of the molecules between the liquid and gel phases. If K is that fraction of the gel accessible to the molecules, then the volume of gel accessible to the molecules is given by VxK. Therefore, the total volume accessible to the molecules in the liquid phase and the fraction of the gel phase is $V = Vo + VxK$

$$\text{Whence } K = \frac{V - Vo}{Vx} = \frac{Ve - Vo}{Vt - Vo}$$

Ve is the elution volume.

Laurent & Killander (1964) have shown that the radius of gyration of the molecules can be directly related to K by the equation

$$K = \exp. \left[\pi L (r_s + r_r)^2 \right]$$

where L is the concentration of the macromolecules constituting the supporting network in the gel solution, r_s is the Stokes radius of the molecule, and r_r is the radius of gyration of the macromolecular rods. Thus, by calibrating a column using proteins of known Stokes radius, it is possible to estimate the Stokes radius of any other molecule after determining its K value.

Bio-Gel A-15m containing 4% agarose (Bio-Rad Laboratories, Richmond, California, U.S.A., Lot no. 6197, Mesh 100-200) was degassed and packed into a 1.5cm diameter glass column to a height of 90cm. This agarose gel is supplied as a suspension containing 0.02% azide. After packing 1M-potassium acetate pH 7.0 was passed through the column until no more azide could be detected by spectrophotometry. The potassium acetate was allowed to pass through the column for a further 24h to ensure complete equilibration and the flow rate adjusted to 6ml/h. 1M-potassium acetate pH 7.0 containing 0.02% (w/v) was flushed through the system after each separation to prevent bacterial degradation.

The total bed volume of the agarose was estimated by filling the column to a height of 90cm with water and measuring the volume required. The void volume was determined

by applying a suspension of narcissus mosiac virus to the column. The size of this virus is such that it is totally excluded from the gel particles. The volume of effluent collected

TABLE 1. Summary of agarose column characterization

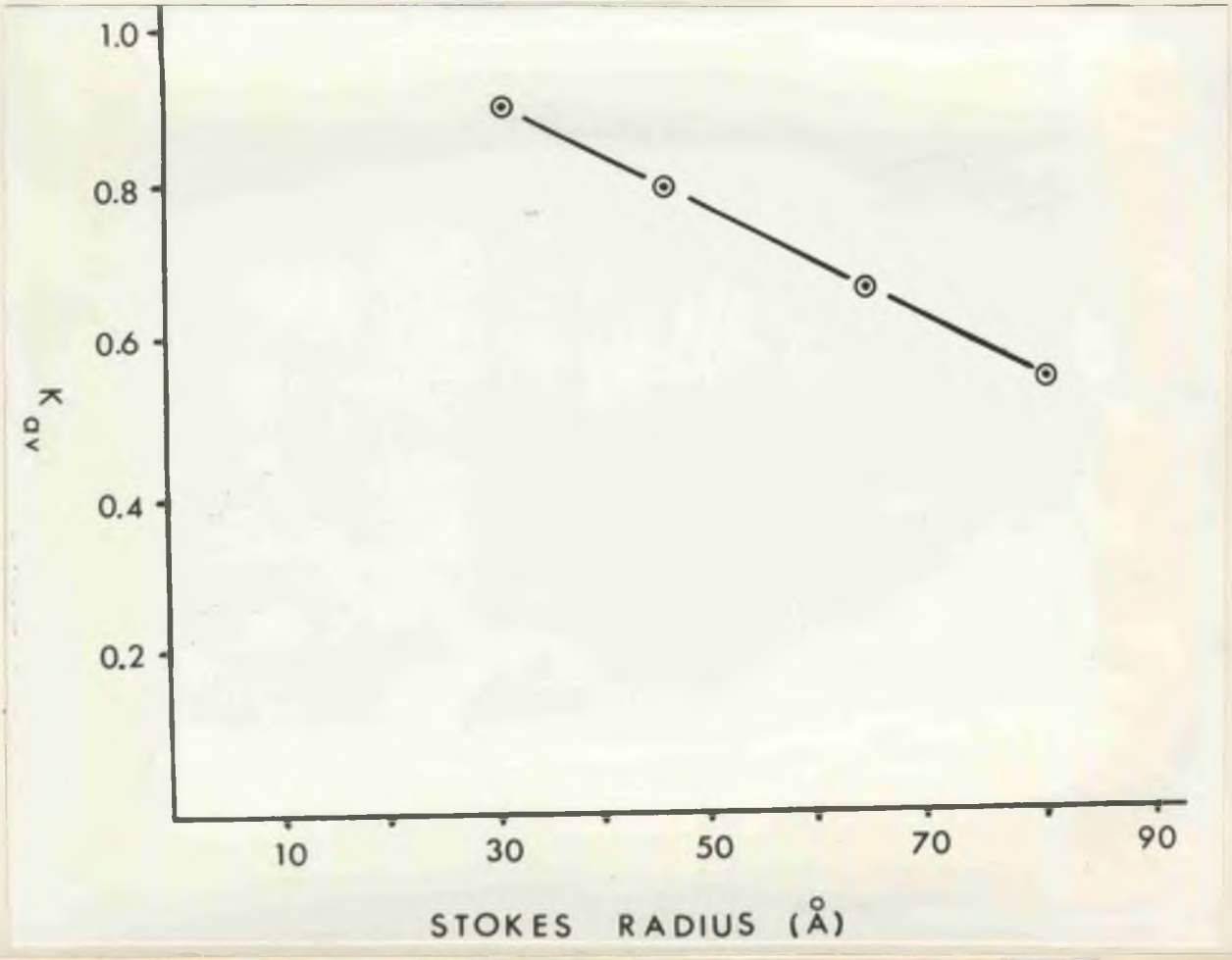
PROTEIN°	Kav	STOKES RADIUS rsÅ
Horse radish peroxidase	0.853	30.2
Bovine thyroglobulin	0.546	81.0+
Glutamic dehydrogenase	0.657	64.0+
Yeast alcohol dehydrogenase	0.783	45.5+

Laurent & Killander (1964)

Rogers & Thompson (1963) +

° All proteins supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A.

Fig. 1. Plot of Kav against Stokes radius



before the virus was excluded from the column, determined by spectrophotometry, was taken to be the void volume of the column. The column was characterized by determining the elution volumes of four proteins of known Stokes radius, namely horse radish peroxidase, bovine thyroglobulin, glutamic dehydrogenase and yeast alcohol dehydrogenase. Each protein was dissolved in 1M-potassium acetate and passed through the column separately. 3ml fractions of effluent were collected and each tube was read at 278 mu in a spectrophotometer. The results of this analysis is summarised in Table 1, and a plot of Kav against Stokes radius in Å units is shown in Fig. 1.

Samples, 10mg each of heparin proteoglycan were dissolved in 2ml 1M-potassium acetate pH 7.0 and applied to the column. The flow rate had previously been adjusted to 12ml/h and the fractions, 3ml each, were analysed for uronic acid content by the method of Bitter & Muir (1962).

TREATMENT OF HEPARIN PROTEOGLYCAN WITH MERCAPTOETHANOL

20mg of dried purified complex was dissolved in 2ml distilled water and adjusted to pH 8.0 with 0.1M-NaOH. 1.56g of 2-mercaptoethanol was added and the volume was made up to 20ml and the pH readjusted. The reaction was allowed to continue at room temperature for 24h under nitrogen. Iodoacetamide equivalent to twice the amount of mercaptoethanol was then added to the reactants and the mixture was shaken for a further 4h. This process was repeated until all the

mercaptoethanol had reacted, verified by the loss of its characteristic odour. The reaction mixture was then dialysed for 24h at 4°C against distilled water, during which time a white suspension formed. This suspension also formed when mercaptoethanol and iodoacetamide were reacted in water, but was found to be soluble in ethanol. The mercaptoethanol treated material was precipitated from solution by the addition of 4 vol. of ethanol and 1 vol. of ethanol saturated with potassium acetate. After the material had been dried, it was redissolved in 2ml 1M-potassium acetate and subjected to column chromatography on agarose

ACRYLAMIDE GEL ELECTROPHORESIS.

Acrylamide gel electrophoresis was performed by the gel slab method of Raymond & Weintraub (1959) as described by Tombs (1965).

15g of "Cyanogum 41" was dissolved in 100ml of distilled water, and filtered to remove any undissolved particles. 0.5ml of 2-dimethylaminopropionitrile followed by 1.0ml of a freshly prepared 10% (w/v) ammonium persulphate solution were then mixed into the clarified "Cyanogum 41" solution. This mixture was poured into a perspex casting tray (8 x 16cm and 2mm deep) containing four perspex spacers, (10 x 0.5 and 2mm high) placed 3cm from one end of the tray. These spacers form the starting slots of the gel. After removal of all air bubbles from the tray, a glass plate was placed on top so that a little of the gel mixture was excluded. This procedure ensured the air-

tight seal essential for gellation. After the gel formed, it adhered to the glass plate, from which it was carefully removed and allowed to dialyse against buffer for 24h.

The gels were run in the discontinuous system of 0.05M-Tris citrate pH 8.6 inside the gel and 0.05M-sodium borate pH 8.6 in the tank. Experiments were carried out with the gels in perspex supporting trays, of the same dimensions as the casting trays but containing no perspex spacer, in a Shandon Universal Electrophoresis Apparatus. This buffer system produces a brown line in the gels, marking the interface of the two buffers, which allows each gel to be terminated at the same point.

Samples of the proteoglycan and heparin (Wilson Laboratories, Chicago, Ill, U.S.A.) were dissolved in 0.05M-tris citrate buffer pH 8.6 and placed in the starting slots of the gels. Double paper wicks of Whatmann 3MM paper were used to connect the gels to the tank and a voltage of 350v applied. The gels were run without cooling until the brown line reached the marker in the running tray, and were then removed and stained with amido black or toluidine blue.

The amido black stain was composed of methanol:water: glycerol:acetic acid (50:50:20:1, by volume) saturated with amido black. The gels were allowed to stain for 20min in this mixture and were destained in 1% acetic acid overnight. The toluidine blue stain was composed of methanol:water: glycerol: (50:50:20, by volume) saturated with toluidine blue.

The gels were stained for 20min and destained with distilled water. The methanol used in the above staining mixtures fixes the samples in the gels, but the use of methanol alone would cause marked shrinkage and cracking. The glycerol in the mixture mediates the effects of the alcohol and prevents these side effects. After destaining, the gels were stored in polythene bags.

The acrylamide gel slab method was used in preference to the disc method (Ornstein & Davis, 1964) because, although slower, the former method allows exact comparison of samples since they are travelling through an identical gel at an identical voltage for an identical time.

AMINO END-GROUP ANALYSIS

The 2-chloro-3,5-dinitropyridine method (Signor et al., 1964) was used since this procedure has the advantage over the 2,4-dinitrofluorobenzene method that the glycine derivative is stable to acid hydrolysis.

A 20mg sample of the heparin proteoglycan was dissolved in 5ml water in the reaction vessel of a Radiometer pH-stat set at pH 9.0. At this pH the only amino acid which will not react with the reagent is tryptophan. The micro-syringe was filled with 0.25M-NaOH and nitrogen was bubbled through the solution. After the pH had been stabilised, 6ml of ethanol containing 6.1mg of 2-chloro-3,5-dinitropyridine was added and the reaction was allowed to proceed for 5h at room temperature. When the reaction was complete, the solution

was acidified to pH 2.0 with HCl and excess reagent and 2-hydroxy-3,5-dinitropyridine were removed by exhaustive extraction with ethyl acetate. The dinitropyridyl materials were then precipitated by the addition of 4 volumes of ethanol and one volume of ethanol saturated with potassium acetate. The precipitate was washed several times with ethanol and dried in a vacuum desiccator. This material was then hydrolysed, after weighing, with 20ml of constant-boiling HCl in sealed tubes under nitrogen at 60°C for 10h. The hydrolysate was adjusted to pH 2.0 with KOH and the liberated dinitropyridyl amino acids were extracted with 6 portions (10ml each) of ethyl acetate. The pooled ethyl acetate extracts were twice washed with 5ml 0.01M-HCl. The water phase together with the acid washings of the ethyl acetate extracts were taken to dryness and the residue was dissolved in constant-boiling HCl and subjected to hydrolysis for 24h at 110°C in sealed tubes under nitrogen prior to amino acid analysis. The identity and concentration of the amino end-group amino acids was established by comparison with the amino acid analysis of untreated material.

ULTRACENTRIFUGATION

To evaluate the homogeneity of the heparin proteoglycan it was dissolved in 0.1M-sodium phosphate buffer pH 7.0, containing 0.4M-NaCl. The material was exhaustively dialysed against this buffer system and submitted to analytical ultra-centrifugation at 59780 rev./min. Its concentration was

determined in a refractometer.

OSMOMETRY

The instrument used was a 501 high-speed standard membrane osmometer (Hewlett-Packard, Pa., U.S.A.) equipped with B-1 membranes (Schleicher & Schnell, Keene, Germany). A temperature of 25°C was maintained throughout the experiment.

All measurements of osmotic pressure were made with a high concentration of diffusible electrolyte to blank out any effect due to counter-ions. The sample was exhaustively dialysed against 1M-potassium acetate buffer pH 7.0, and, after dialysis, the protein-polysaccharide concentrations were determined using a differential refractometer (Bendix Electronics, Nottingham) using the 546 mu mercury line. No loss of material was detected during dialysis.

A series of progressive dilutions of the sample was prepared and four determinations of osmotic pressure were made for each sample concentration. A graph of π/C as a function of C (where C = concentration and π = osmotic pressure) was plotted.

ELECTRON MICROSCOPY

HEPARIN PROTEOGLYCAN.

The heparin proteoglycan was isolated as previously described. The white precipitate which formed during the first precipitation with alcohol and potassium acetate was collected by centrifugation and dried in acetone. A sample

of this precipitate (sample 1) was submitted to electron microscopic analysis. When this material was resuspended in water, 40% remained insoluble. The soluble fraction was twice subjected to alcohol precipitation and a sample of the final dry powder (sample 2) was dissolved in water for examination in the electron microscope.

The aqueous solutions of samples 1 and 2 were exhaustively dialysed against distilled water and sprayed at a concentration of approximately 3ug/ml on to carbon-coated grids. The monolayers of the heparin-protein complex were then stained for 10min in 0.5% bismuth nitrate in 0.1M-nitric acid (Serafini-Fracassini & Smith, 1966).

MAST CELLS.

Rat peritoneal fluid was collected using a modification of the method described by Uvnäs & Thon (1959). The rats were decapitated and exsanguinated. Six ml of phenol red-free Hank's solution were then injected into the peritoneal cavity and, after the abdomen had been gently massaged for 90sec, the peritoneal fluid was removed, great care being taken to ensure that no blood was allowed to enter the peritoneal cavity; if contamination, due to leakage of blood, was observed the samples were discarded. The isolated peritoneal fluid was centrifuged for 10min at 450g, and the cell pellet was washed with phenol red-free Hank's solution, fixed in 1% osmium tetroxide in veronal-acetate buffer pH 7.2

and embedded in Araldite. Ultrathin sections were cut with glass knives on a LKB-111 ultra-microtome and stained with bismuth nitrate or uranyl acetate. In trial experiments gluteraldehyde was used but was found to impair the bismuth staining.

BISMUTH STAIN

The bismuth stain was prepared by the method of Serafini-Fracassini & Smith (1966).

1g bismuth nitrate was dissolved in 10ml 2M-nitric acid and this was made up to a final volume of 200ml with distilled water. Since there is a tendency for the bismuth nitrate to precipitate during dilution, the water was allowed to drip slowly into solution from a burette while the bismuth nitrate in nitric acid solution was agitated with a magnetic stirrer. This procedure gives a 0.5% solution of bismuth nitrate in 0.1M-nitric acid pH 1.2. Staining times of 10min were used.

URANYL ACETATE STAIN

Grids were stained in 2% uranyl acetate (w/v) for 20mins.

STAINING PROCEDURE

Grids were stained by flotation on the surface of the stains in staining dishes. After the required time had elapsed, the staining dishes were filled with water and the grids were washed for 30mins. This washing procedure was carried out several times.

RESULTS

ISOLATION PROCEDURE

The material, after the initial ethanol fractionation, was dried in acetone and stored in a desiccator. When a sufficient quantity for analysis had been collected, the pooled material was redissolved in water and resubmitted to ethanol fractionation. It was found that 40% of this first preparation remained insoluble after 12h stirring. This insoluble fraction was found to have a sulphur content of only 0.25%. The soluble fraction, however, once precipitated and dried, gave a preparation that was readily soluble. Its yield averaged 35mg of protein-polysaccharide per 100g of dry ox liver capsule. This is the fraction that was subjected to chemical and biological analysis.

BIOLOGICAL ACTIVITY

The anticoagulant activity of the preparation averaged 55 B.P. units/mg.

PROTEIN CONTENT AND AMINO ACID COMPOSITION OF THE PROTEOGLYCAN OF HEPARIN

To determine the protein content of the heparin proteoglycan and individual amino acid recoveries after acid hydrolysis, a sample of the material was analysed after hydrolysis for 24, 36 and 72h at 110°C.

The results of amino acid analysis are reported in Table 2, column (A), where the concentrations are expressed as ug of anhydro amino acid/10mg of dry and ash-free material.

The corrected concentrations of the various amino acids were calculated by either selecting the greatest value for those showing increased concentration during hydrolysis or by extrapolating to zero hydrolysis time those values showing linear kinetics of hydrolytic destruction. In the case of serine and threonine all three values gave a linear relationship, but since non-linear behaviour was observed for proline, tyrosine and arginine + ornithine only the 24 and 36h values were extrapolated to zero hydrolysis time. In Table 2, column (B), the corrected amino acid composition of the protein component is reported, whereas column (C) shows the percentage zero time recovery for each amino acid after 24h hydrolysis. A protein content of 13.21% was derived by summation of the corrected values.

ANALYSIS OF THE CARBOHYDRATE MOIETY

The results of analysis for hexosamine content are reported in Table 3. A glucosamine to galactosamine ratio of 26.4 to 1 was obtained.

The total hexuronic acid content is reported in Table 3, expressed both as free acid and potassium salt.

The quantitative determination of xylose showed that more than one chromogen was formed during the reaction of the anthrone and the heparin proteoglycan. The main one had an absorption maximum at 615mu, but at least another two were present, with absorption maxima at 560 and 505mu. The optical density was read at 615mu and the xylose content is reported

in Table 3.

Thin-layer chromatography for neutral sugars revealed only two spots, after staining with anisaldehyde in sulphuric acid. These spots produced the colours and Rf values expected of xylose and galactose. These were the only neutral sugars present in the hydrolysates.

The sulphur content, determined by the method of Giellman & Tötig (1960), is reported in Table 3, where it is expressed as free sulphur and ester sulphate, potassium salt.

ALKALI-INDUCED β-ELIMINATION

The occurrence of β-carbonyl elimination in a sample of heparin proteoglycan was demonstrated by recording, over 24h, the change in absorbance of the preparation of 241mu, when dissolved in 0.5M-NaOH (Fig. 2a). A sample of bovine chondro-mucoprotein was subjected to identical treatment and its reaction plot is reported for comparison (Fig. 2b).

The amino acid compositions of the two samples subjected to alkaline treatment either with or without catalytic hydrogenation are shown in Table 4, where they are compared with the corresponding hydrolysate of untreated material. It can be seen that β-elimination, both with and without hydrogenation, results in a reduction in the threonine concentration, by 19.1% and 33.8% respectively, while the serine content is only very slightly affected. Furthermore after alkaline treatment in the presence of Adams catalyst,

TABLE 2. Amino acid analysis of heparin proteoglycan

(*A*) Wt. of anhydro amino acid (μg./10mg. of heparin–protein complex, ash- and moisture-free); (*B*) wt. of anhydro amino acid (g./100g. of the protein component); (*C*) recovery after 24hr. hydrolysis (%).

Time of hydrolysis (hr.) ... ... / Amino acid	(*A*) 24	(*A*) 36	(*A*) 72	(*A*) Corrected values	(*B*) Corrected values	(*C*)
Cysteic acid	62·3	60·4	67·3	63·3*	4·79	98·3
Hydroxyproline	1·5	Trace	Trace	1·5†	0·11	100·0
Aspartic acid	128·8	128·6	128·6	128·8†	9·75	100·0
Threonine	64·9	61·8	51·6	71·7‡	5·43	90·5
Serine	53·9	49·0	33·5	64·3‡	4·87	83·8
Glutamic acid	183·5	190·3	191·2	191·2†	14·48	96·0
Proline	80·9	76·5	75·9	89·7§	6·79	90·2
Glycine	83·7	85·8	88·3	88·3†	6·69	94·8
Alanine	73·9	75·2	80·0	80·0†	6·06	92·4
Valine	66·6	68·6	68·8	68·8†	5·21	96·8
Methionine	0·0	0·0	0·0	0·0	0·00	
Isoleucine	40·6	42·2	42·7	42·7†	3·23	95·1
Leucine	105·7	109·5	109·3	109·5†	8·29	96·5
Tyrosine	6·3	4·2	4·6	10·5§	0·79	60·0
Phenylalanine	69·5	70·7	68·6	70·7†	5·35	98·4
Hydroxylysine	0·0	0·0	0·0	0·0	0·00	
Ornithine	5·1	5·1	6·2			
Lysine	111·0	112·2	112·2	112·2†	8·49	98·9
Histidine	31·2	31·3	28·7	31·3†	2·37	99·7
Arginine	90·3	89·9	82·0		7·29	
Arginine + ornithine	(95·4)	(95·0)	(88·2)	96·3§		99·1
Totals	1259·7	1261·3	1239·5	1320·8	99·99	

* Average value (24hr., 36hr. and 72hr. hydrolysates).
† Greatest value.
‡ Extrapolation to zero time (24hr., 36hr. and 72hr. hydrolysates).
§ Extrapolation to zero time (24hr. and 36hr. hydrolysates).

TABLE 3. Composition of heparin proteoglycan

Values are expressed as percentages of dry ash-free material.

Protein		13·21
Total hexosamine (as free base)		14·82
Glucosamine	14·28	
Galactosamine	0·54	
Hexuronic acid (as free acid)	27·83	
Hexuronic acid (as potassium salt)		33·39
Xylose		0·68
Sulphur (as S)	8·90	
Sulphur (as ester sulphate, potassium salt)		33·09
Total		95·19

the glycine content is reduced by only 12.9%, whereas the reduction in the concentrations of alanine, leucine, isoleucine and valine averages 28.0%. These figures are interpreted as reflecting an increase in glycine content.

Owing to the superimposition of glucosamine and α-aminobutyric acid peaks, precise identification of the latter in hydrolysates of the material subjected to β-elimination and hydrogenation proved unfeasible by automatic amino acid analysis, although the marked asymmetry of the glucosamine peak implied its presence. However, when two-dimensional thin layer chromatography for amino acids was applied to this hydrolysate an extra spot in the pattern was observed. The material contained in this spot was found to co-chromatograph with a standard of α-aminobutyric acid. Thus, the presence of α-amino butyric acid in the hydrolysates of heparin proteoglycan subjected to β-elimination and hydrogenation was conclusively demonstrated.

COLUMN CHROMATOGRAPHY ON AE-CELLULOSE

Column chromatography of commercial heparin and chondroitin sulphate was performed on AE-cellulose as described in the Materials and Methods section. Chondroitin sulphate and heparin were eluted by 1.0M-and 1.5M-sodium chloride respectively. In this system, however, concentrations of sodium chloride in excess of 3M failed to displace any fraction of the untreated material. After β-elimination,

Fig. 2. β-elimination

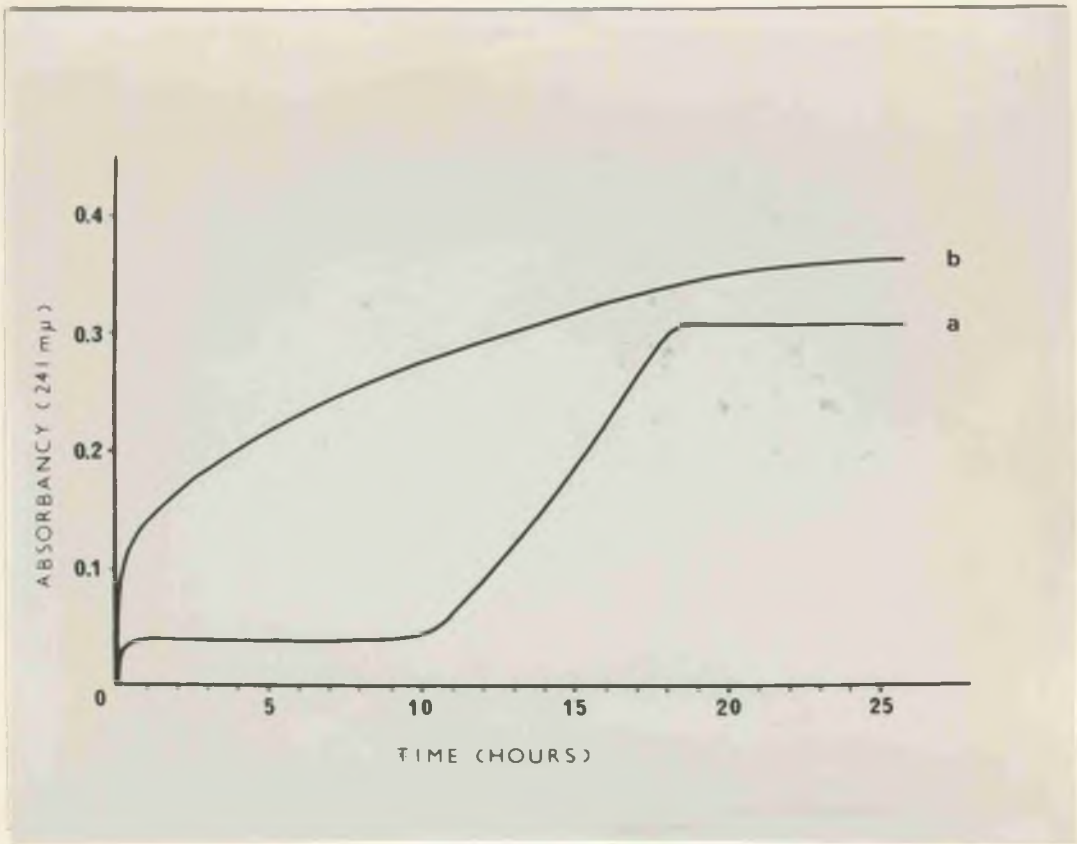


Fig. 2.

Assay a: 360mg/ml heparin proteoglycan in 0.5M-NaOH compared to an aqueous solution of identical concentration as blank.

Assay b: 540mg/ml chondroitin sulphate proteoglycan in 0.5M-NaOH compared to an aqueous solution of identical concentration as blank.

The absorbancy at 241mu was recorded with a Beckman D.B. recording spectrophotometer.

however, the polysaccharide was eluted by 1.5M-sodium chloride.

ULTRACENTRIFUGATION

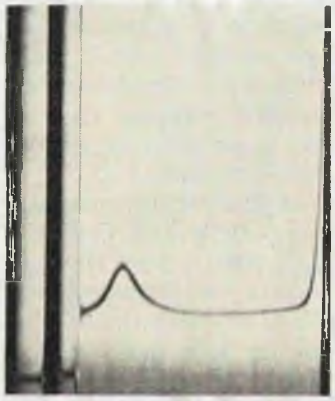
To evaluate the homogeniety of the preparation, it was dissolved in 0.1M-sodium phosphate buffer pH 7.0, containing 0.4M-NaCl, and submitted to analytical ultracentrifugation at 59780 rev./min. In this system the material behaved as a single peak characterised by a sedimentation coefficient of 1.85S. (Fig. 3.).

TABLE 4. Amino acid analysis of heparin proteoglycan after β-elimination

Values are reported as wt. of anhydro amino acid (μg./10 mg. of dry ash-free protein–polysaccharide complex). All hydrolyses were carried out for 24 hr. (*A*) Untreated sample; (*B*) sample after β-elimination; (*C*) sample after β-elimination and reduction in the presence of Adams catalyst. N.D., Not determined.

Amino acid	(*A*)	(*B*)	(*C*)
Cysteic acid	62·3	N.D.	N.D.
Hydroxyproline	1·5	0·0	0·0
Aspartic acid	128·8	120·4	89·3
Threonine	64·9	52·5	43·0
Serine	53·9	52·6	50·8
Glutamic acid	183·5	182·7	148·2
Proline	80·9	80·5	62·3
Glycine	83·7	83·8	72·9
Alanine	73·9	76·1	57·0
Valine	66·6	64·3	47·1
Methionine	0·0	0·0	0·0
Isoleucine	40·6	37·7	29·1
Leucine	105·7	99·9	72·4
Tyrosine	6·3	32·7	35·2
Phenylalanine	69·5	65·6	45·1
Hydroxylysine	0·0	0·0	0·0
Ornithine	5·1	9·0	15·3
Lysine	111·0	105·5	72·1
Histidine	31·2	26·6	24·9
Arginine	90·3	86·7	57·3
Arginine + ornithine	(95·4)	(95·7)	(72·6)

Fig. 3. Ultracentrifugal pattern of heparin proteoglycan. Its total concentration was 0.945% (w/v). The picture was taken at a schlieren angle of 60° at 80 min after the rotor had reached a nominal speed of 59780 rev./min. The direction of sedimentation is from left to right.



COLUMN CHROMATOGRAPHY ON AGAROSE

Column chromatography of the heparin proteoglycan was carried out in 1M-potassium acetate pH 7.0 in a pre-calibrated Bio-Gel A-15m column (See Materials and Methods).

The elution pattern of material subjected to this process is shown in Fig. 4. The column effluent was monitored for hexuronic acid content, and from these values the amount of heparin proteoglycan present in each fraction was calculated, together with the total recovery of material from the column. Four fractions were recovered characterised by elution volumes of 48, 58, 93 and 117 ml respectively, containing 2, 3, 15, and 80% of the recovered sample. The percentage recovery of the heparin proteoglycan averaged 98%

throughout. By substituting the above elution volumes into the standard equation, Kav for each fraction, except the first, (which was excluded) can be calculated and, from the standard graph of Kav against Stokes radius, an approximate value of Stokes radius for each fraction is obtained. In the case of the heparin proteoglycan the fractions F II, III and IV, are characterized by Stokes radii of approx. 179Å, 94Å and 32.5Å respectively.

It can be seen from the elution pattern that the greatest part of the material is contained in F IV. This fraction was collected, precipitated with ethanol, dried in acetone and stored in a desiccator for further analysis.

In view of the appreciable cystine or cysteine content of the original material, it was decided to subject a sample to treatment with 2-mercaptoethanol, in the hope of resolving the role of possible disulphide bridges in the macromolecules.

After 24h treatment with 2-mercaptoethanol and subsequent blocking of the reduced thiol groups with iodoacetamide, the elution pattern of a sample subjected to column chromatography on Bio-Gel A-15m remained identical to that observed for untreated samples (Fig. 4). A sample of pooled F I and F II from previous analyses was also treated in the above manner. The elution pattern of this material revealed that in addition to unaffected F I and F II a small amount of F III and F IV was also present.

ACRYLAMIDE GEL ELECTROPHORESIS OF HEPARIN PROTEOGLYCAN

The separation pattern of the heparin proteoglycan was examined over a wide pH range in 7.5% polyacrylamide gels. The pattern (Fig. 5) was very similar to the elution diagram obtained from separation on agarose. The majority of the material migrated as a single peak close to the solvent front, although a certain degree of polydispersity was apparent. A portion of material which failed to move into the gel can be seen at the origin slots. This fraction is probably of around the same dimensions as F I and F II obtained from agarose chromatography. When the gels were stained with toluidine blue, the entire electrophoretic pattern showed intense metachromasia, but no orthochromatic bands were observed. On the other hand, in gels stained with amido black, there was no evidence for the presence of free protein.

AMINO ACID COMPOSITION OF HEPARIN PROTEOGLYCAN FRACTION F IV

The results of amino acid analysis of a 24h hydrolysate of the heparin proteoglycan fraction F IV, obtained by chromatography on agarose, is reported in Table 5, column (B), where the concentrations are expressed as ug of amino acid anhydro amino acid/10 mg of dry and ash-free material. Column (A) reports the 24h hydrolysis values for the total extract (See Table 2). A protein content of 13.36% was derived for F IV from the corrected amino acid values.

Fig. 4. Elution pattern of heparin proteoglycan on agarose

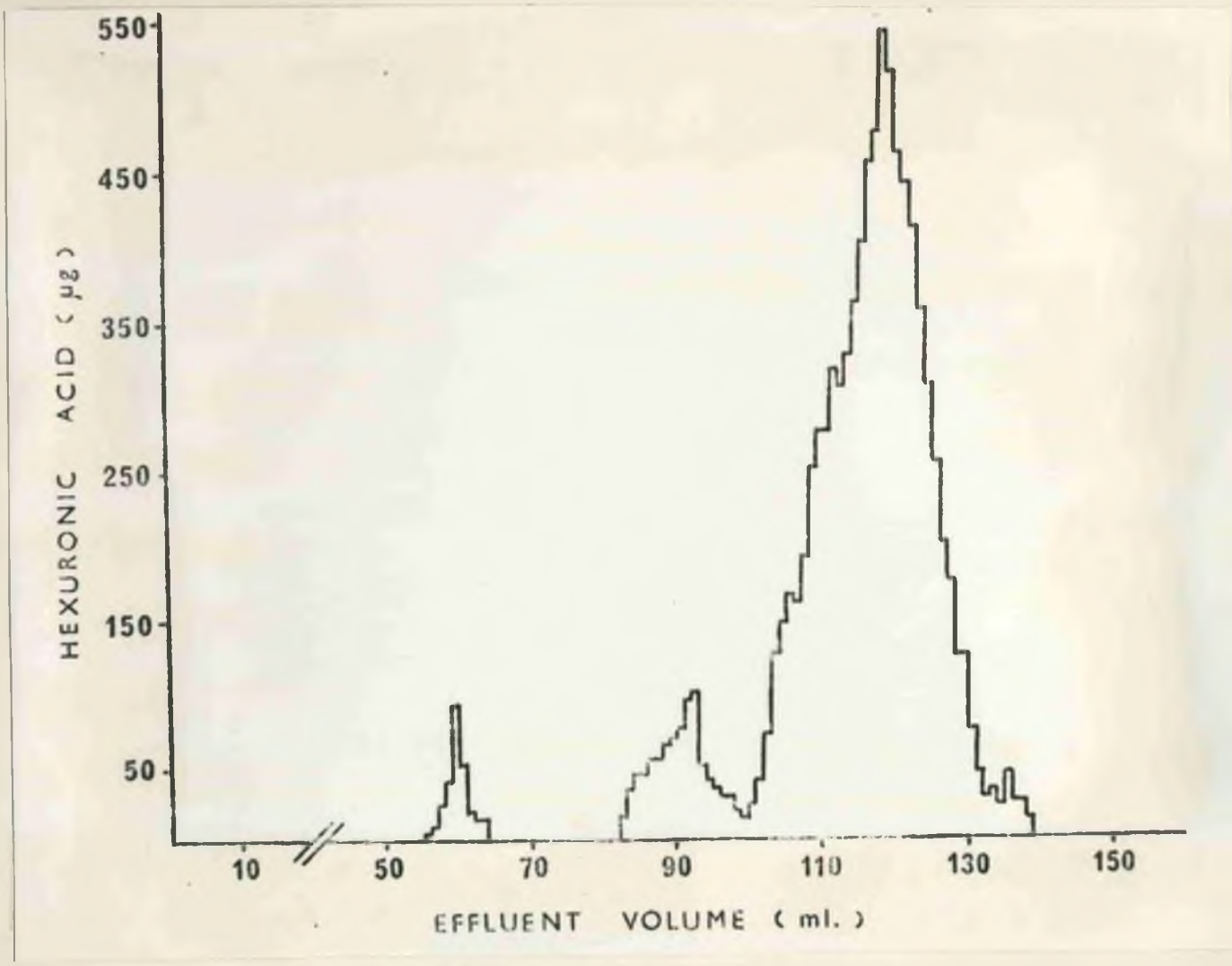


Fig. 4. Column: 90cm x 1.5cm, Bio-Gel A-15m. Solvent: 1M-potassium acetate pH 7.0. The effluent was analysed for hexuronic acid content by the method of Bitter & Muir (1962).

Fig. 5. Electrophoretic pattern of the heparin proteoglycan

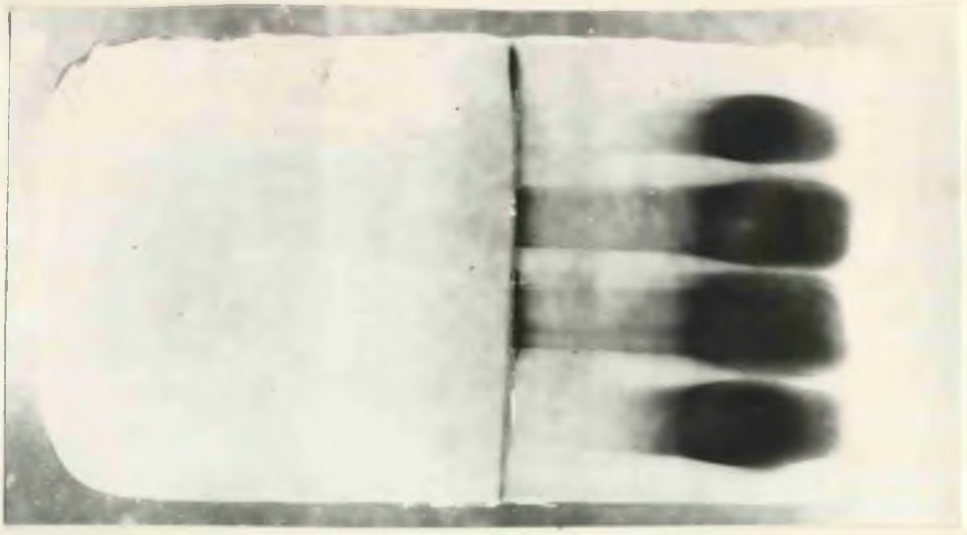


Fig. 5. 7.5% acrylamide gel. Tris-citrate/sodium borate discontinuous buffer pH 8.6. Stain: toluidine blue. Central slots: heparin proteoglycan. Outer slots: heparin (Wilson Laboratories)

Table 5. Amino acid analysis of heparin proteoglycan fraction F IV.

(A) (B) wt of anhydro amino acid after 24h hydrolysis (ug/10mg of ash-and moisture-free material). (A) heparin proteoglycan (B) fraction F IV (C) wt of anhydro amino acid after 24h hydrolysis following treatment with 2-chloro-3,5-dinitropyridine (ug/10mg of ash-and moisture-free material).

Amino acid	(A)	(B)	(C)
Cysteic acid	62.3	57.5	57.7
Hydroxyproline	1.5	1.7	1.5
Aspartic acid	128.8	128.8	106.2
Threonine	64.9	64.2	57.2
Serine	53.9	57.9	58.5
Glutamic Acid	183.5	185.1	171.5
Proline	80.9	80.5	80.1
Glycine	83.7	124.2	123.6
Alanine	73.9	73.4	73.4
Valine	66.6	67.0	66.5
Methionine	0.0	0.0	0.0
Isoleucine	40.6	40.9	39.6
Leucine	105.7	91.2	76.1
Tyrosine	6.3	30.0	8.4
Phenylalanine	69.5	69.3	57.5
Hydroxylysine	0.0	0.0	0.0
Ornithine	5.1	6.0	15.6
Lysine	111.0	90.9	61.1
Histidine	31.2	17.9	17.2
Arginine	90.3	89.8	79.5
Arginine + Ornithine	95.4	95.8	95.1
TOTAL.	1259.7	1274.3	

ANALYSIS OF CARBOHYDRATE MOIETY OF HEPARIN PROTEOGLYCAN FRACTION F IV

The results of quantitative analysis for hexosamine, hexuronic acid, xylose and sulphur are reported in Table 6.

During the reaction of the heparin proteoglycan with anthrone to determine the xylose content, only one chromogen was formed, with a peak at 615 mu.

If it is assumed that heparin is joined to protein only through linkages involving xylose, then that weight of heparin which contains 1 mole of xylose will be the molecular weight of the heparin chains. A molecular weight of 11300 is obtained in this way for the heparin chains, based on a xylose content of 1.08% for the heparin proteoglycan.

Table 6. Chemical analysis of heparin proteglycan fraction F IV

Values are expressed as percentages of dry-and ash-free material.

Protein		13.36
Total hexosamine (as free base)		15.42
Hexuronic acid (as free acid)	26.67	
Hexuronic acid (as potassium salt)		32.00
Xylose		1.08
Sulphur (as S)	8.90	
Sulphur (as ester sulphate, potassium salt)		33.09
TOTAL		94.95

AMINO-END GROUP ANALYSIS OF HEPARIN PROTEOGLYCAN FRACTION F IV

A sample (20mg) of the heparin proteoglycan, after treatment with 2-chloro-3,5-dinitropyridine was hydrolysed in constant-boiling HCl at 60°C for 10h. This hydrolysate was exhaustively washed with ethyl acetate to remove the liberated dinitropyridyl-amino acids. The ethyl acetate extracts were washed with dilute acid, and the washings together with the original hydrolysate were further hydrolysed in constant-boiling HCl at 110°C for 24h. The amino acid analysis of the resultant hydrolysate is reported in Table 5, column (C), where it is compared with a 24h hydrolysate of untreated material (Column B). By this method the N-terminal amino acids were identified, and their concentrations determined. Table 7, shows the four N-terminal amino acids together with their concentrations which are expressed as moles of amino acid per 10^6g of protein-polysaccharide.

The number average molecular weight of a protein is that weight which contains one mole of N-terminal amino acid. By this method a molecular weight for the protein-polysaccharide of 19500 was obtained.

The ethyl acetate extracted dinitropyridyl amino acids were not subjected to thin layer chromatography, since, in view of the low concentration and tendency for the production of artifacts during chromatogram development, it was considered that the amino acid difference method was a great deal more reliable.

MOLECULAR WEIGHT DETERMINATION BY OSMOMETRY OF HEPARIN PROTEOGLYCAN FRACTION F IV

The osmotic pressure determinations of F IV was carried out at two concentrations of added HCl in order to check if the contribution of the Donnan distribution of mobile ions was affecting the observed values. The plot of the reduced osmotic pressures (π/C) as a function of solute concentrations (C) is reported in Fig. 6. Because no obvious curvature was detectable correction for non-ideality was performed by linear extrapolation of π/C against C values to C = O by the method of least squares. The results were then fitted to the standard equation.

$$\bar{M}n = \frac{RT}{(\pi/c)_{c \longrightarrow 0}}$$

Where RT was corrected to account for the density of the solvent. A derived number average molecular weight of 20600 was obtained.

A summary of the molecular weight of the heparin proteoglycan determined by osmometry and N-terminal analysis together with the molecular weight of the heparin chain derived from its xylose content is reported in Table 8.

TABLE 7. End-group analysis.

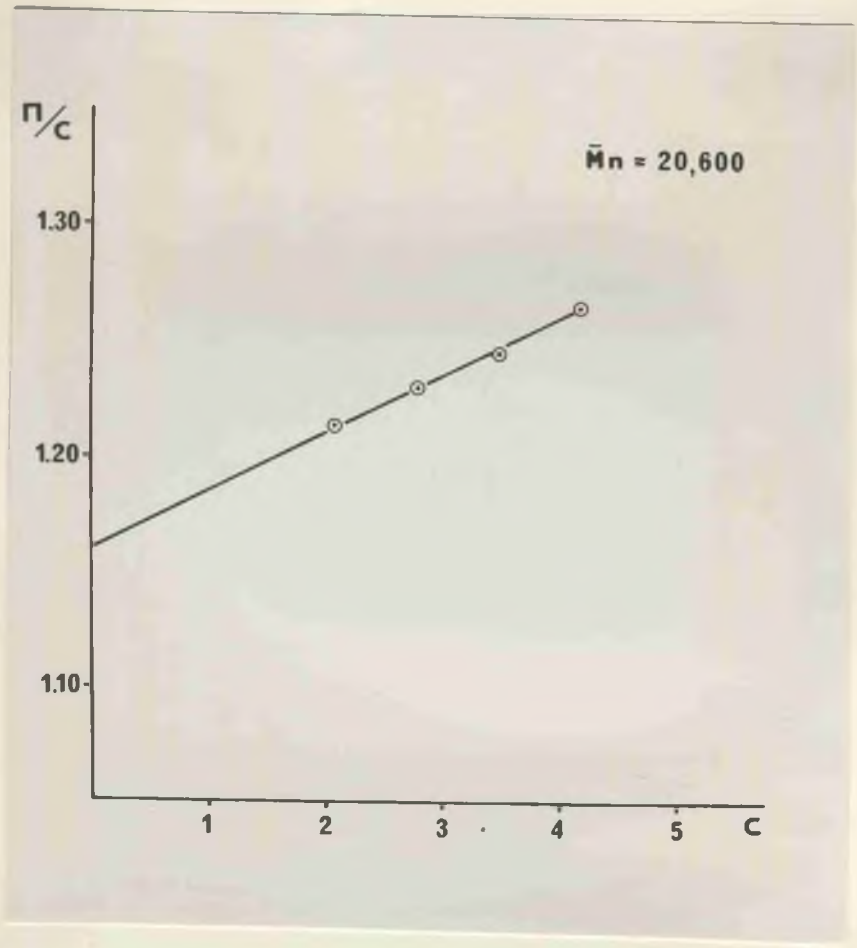
Values are expressed as moles of amino acid/10^6g of protein-polysaccharide

Aspartic acid	19.6
Glutamic acid	10.5
Leucine	13.3
Phenylalanine	8.0
TOTAL	51.4.

TABLE 8. Molecular Weights

Method	Component	Molecular weight
Osmometry	heparin proteoglycan	20600
N-terminal Analysis	heparin proteoglycan	19500
Xylose content	heparin chain	11300

Fig. 6. Plot of reduced osmotic pressure against concentration for the heparin proteoglycan



ELECTRON MICROSCOPY

HEPARIN PROTEOGLYCAN

The electron microscopic appearance of the heparin proteoglycan collected after the first alcohol precipitation (Sample 1) is shown in Plate 1. Some of the material appears as filaments, although it is evident that these filaments represent only a proportion of the total sample, the greater part consisting of short segments or single particles which in some areas have joined together to form structureless aggregates.

When these filaments are examined at a higher magnification (Plate 2), it is evident that they are composed of particles. The average diameter of these particles when arranged in filaments (arrows a, Plate 2) is 35Å. In certain restricted areas, however, the filaments tend to become entangled (arrows b, Plate 2) and the particle diameter averages 50Å. By analogy with the results of an investigation of the morphology of the isolated protein-polysaccharide complex of bovine nasal septum using bismuth nitrate stain (Serafini-Fracassini & Smith, 1966), the 35Å particles are interpretated as single heparin chains which have adopted a coiled cofiguration due to the neutralization of the charges on the sulphate groups by bismuth ions. The larger particles which are formed when the filaments become entangled are probably due to the cross-linking and coiling together of several adjacent heparin chains. Under these conditions,

conditions, therefore, there is no relationship between particle diameter and molecular weight.

The appearance of the final soluble preparation (Sample 2), is shown in Plate 3. Particles exhibiting considerable variation in diameter are present. However, particles of 35Å average diameter are evident, either isolated or arranged as short rows (arrows a). It should be pointed out that the filaments observed in Sample 1 were never found in specimens of the highly purified material.

MAST CELL GRANULES.

URANYL ACETATE STAINED MAST CELL GRANULES.

The electron microscopic appearance of a typical mast cell granule stained with uranyl acetate is shown in Plate 4. It exhibits a uniform particulate appearance, but due to its electron density none of its detailed substructure can be observed. The compound granule (Plate 5) exhibits, for the greater part, an identical appearance. However, one region of its matrix appears to be more heavily stained, giving the impression of a more compact nature.

The disrupted granule shown in Plate 6 is also stained with uranyl acetate. A proportion of the granules contents seems to have been lost revealing a network of electron dense "bodies"

Plate 1

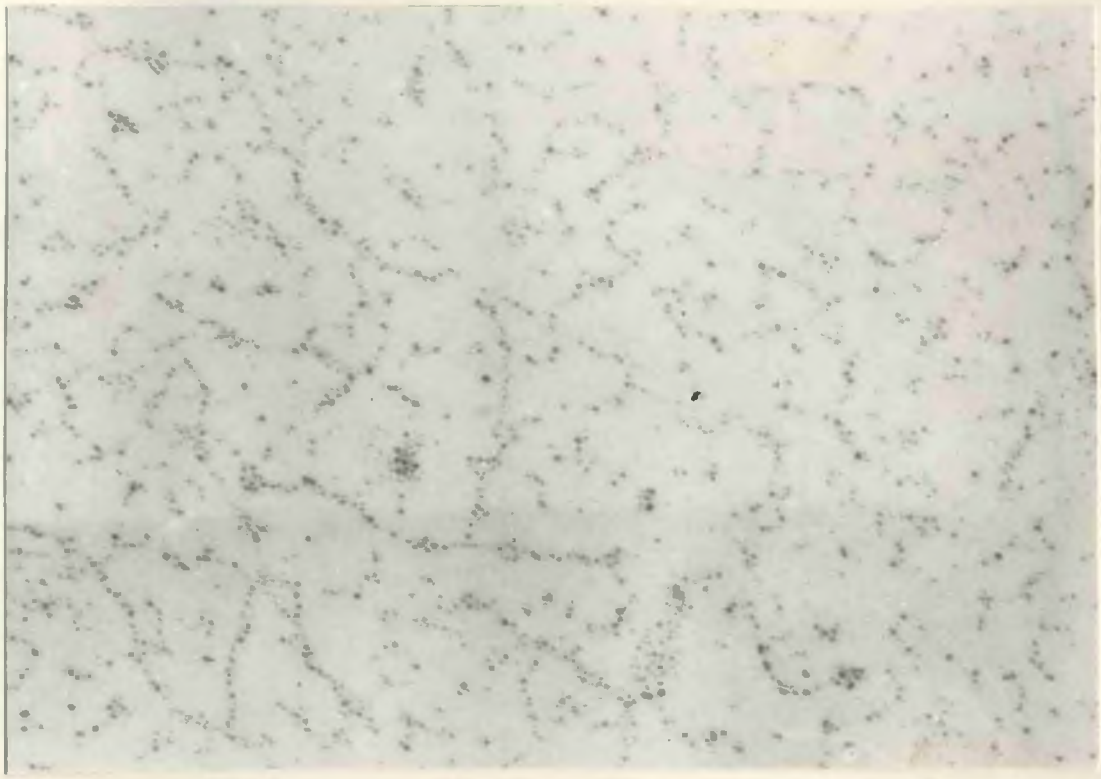


Plate 1

Monolayer of heparin proteoglycan macromolecules sprayed on to the grid after the first alcohol precipitation (Sample 1) and stained with 0.5% bismuth nitrate in nitric acid, pH 1.2. x 177500.

Plate 2

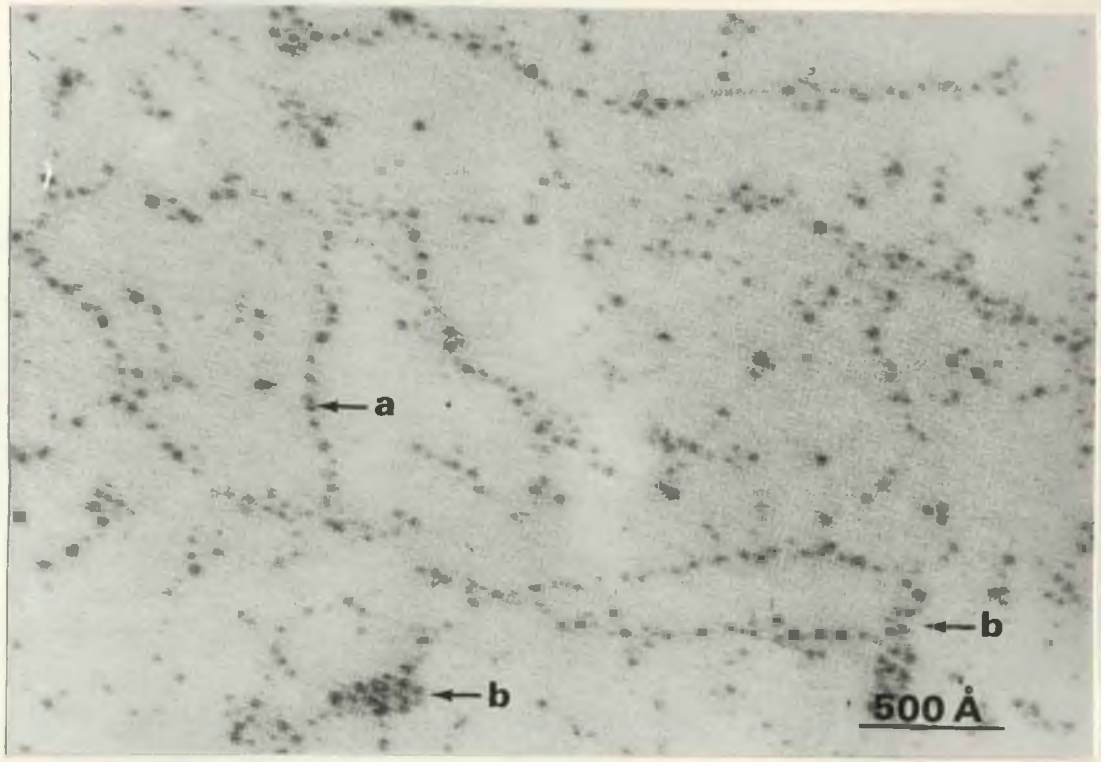


Plate 2

Monolayer of heparin proteoglycan macromolecules sprayed on to the grid after the first alcohol precipitation (Sample 1). Arrow a indicates a typical beaded filament composed of 35-Å particles. Areas of entanglement are evident (arrows b). The stain is 0.5% bismuth nitrate in nitric acid, pH 1.2 x 340000.

Plate 3

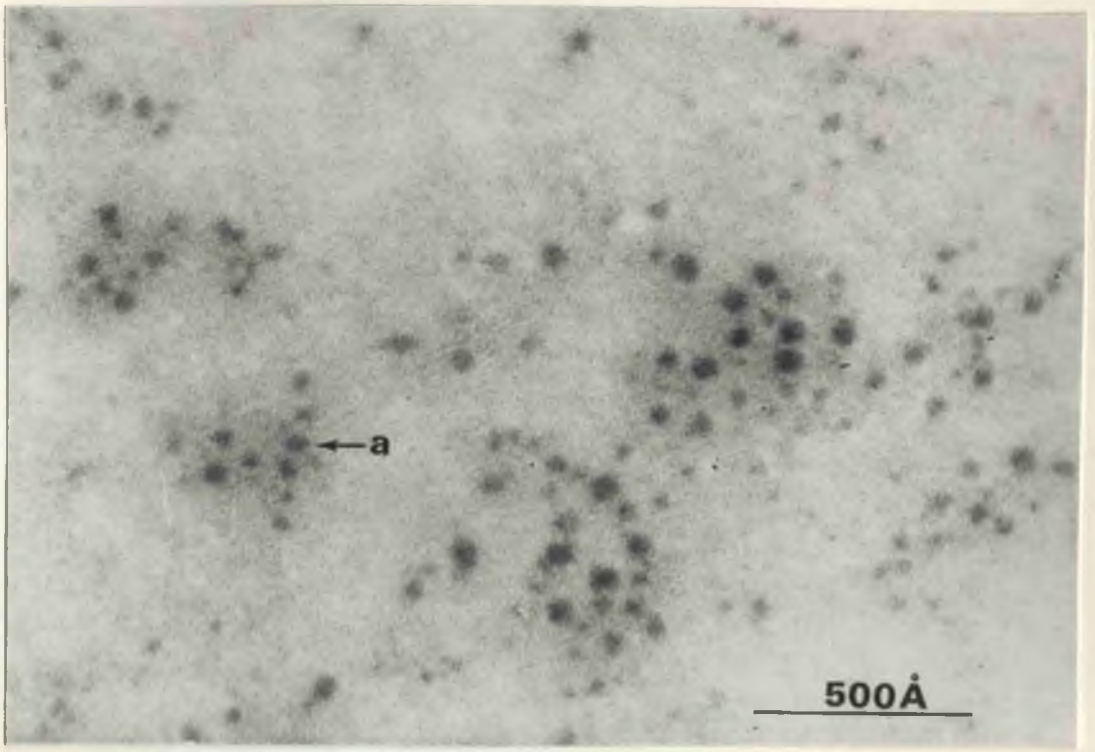


Plate 3

Monolayer of heparin proteoglycan macromolecules after repeated alcohol fractionation (Sample 2). Arrow a points to a short beaded segment composed of 35-Å particles. The stain is 0.5% bismuth nitrate in nitric acid, pH 1.2. x 520000.

connected by material which is less heavily stained.

Uranyl acetate is a non-specific stain when used at neutral pH. At pH 7.0, it would be expected that the uranium ions would react, not only with the strongly acidic groups of any heparin, but also with the many protein components known to be present in the mast cell granules. Although examination of a disrupted granule produces some improvement in the resolution of the substructure of the granule, it remains impossible to make any meaningful deductions.

BISMUTH NITRATE STAINING.

The bismuth nitrate in nitric acid electron microscope stain pH 1.2 is highly specific for strong acid groups with a pK of less than 2.0, that is phosphate and sulphate groups in biological systems. Moreover, since the pK of phosphate groups are very near to 2.0, these stain only relatively lightly. Furthermore, since this reagent does not stain proteins, it results in "clear" areas in cells which have low concentrations of phosphate and sulphate groups. This effect often gives the impression of an apparent loss of protein from the cells which is occasionally confused with poor fixation.

In an electron micrograph of a lymphocyte stained with bismuth (Plate 7) this effect is clearly observed. It is also evident that the nucleus, due to its much higher phosphate ion content, stains more intensely than the other cytoplasmic inclusions, except the granules.

Plate 4

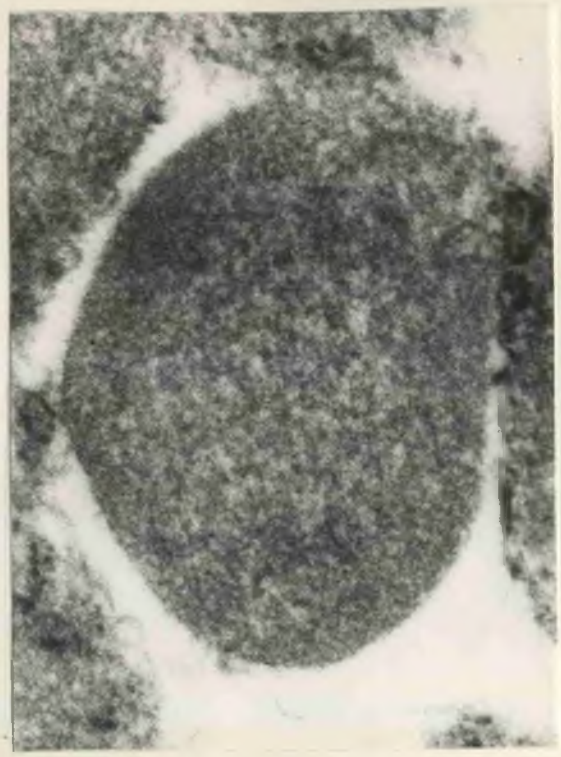


Plate 5

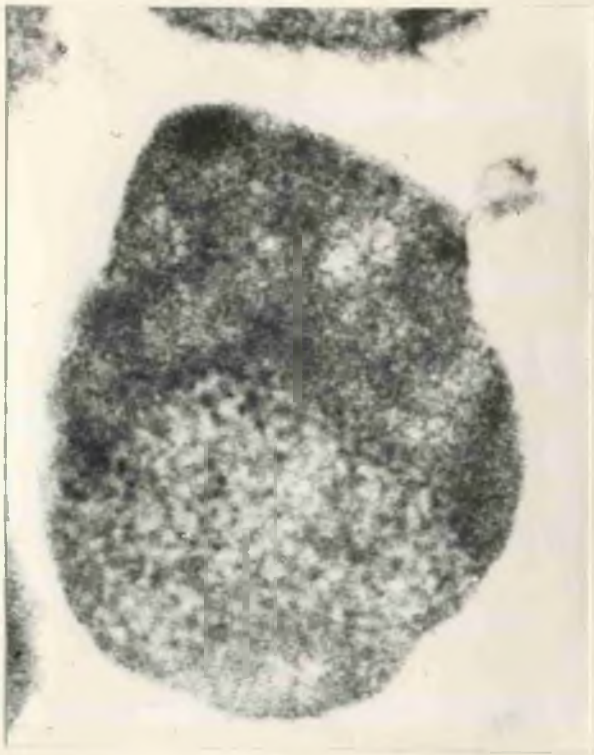


Plate 4

Rat peritoneal mast cell. Simple granule stained with uranyl acetate. x 83000.

Plate 5

Rat peritoneal mast cell. Compound granule stained with uranyl acetate. x 77500.

Plate 6

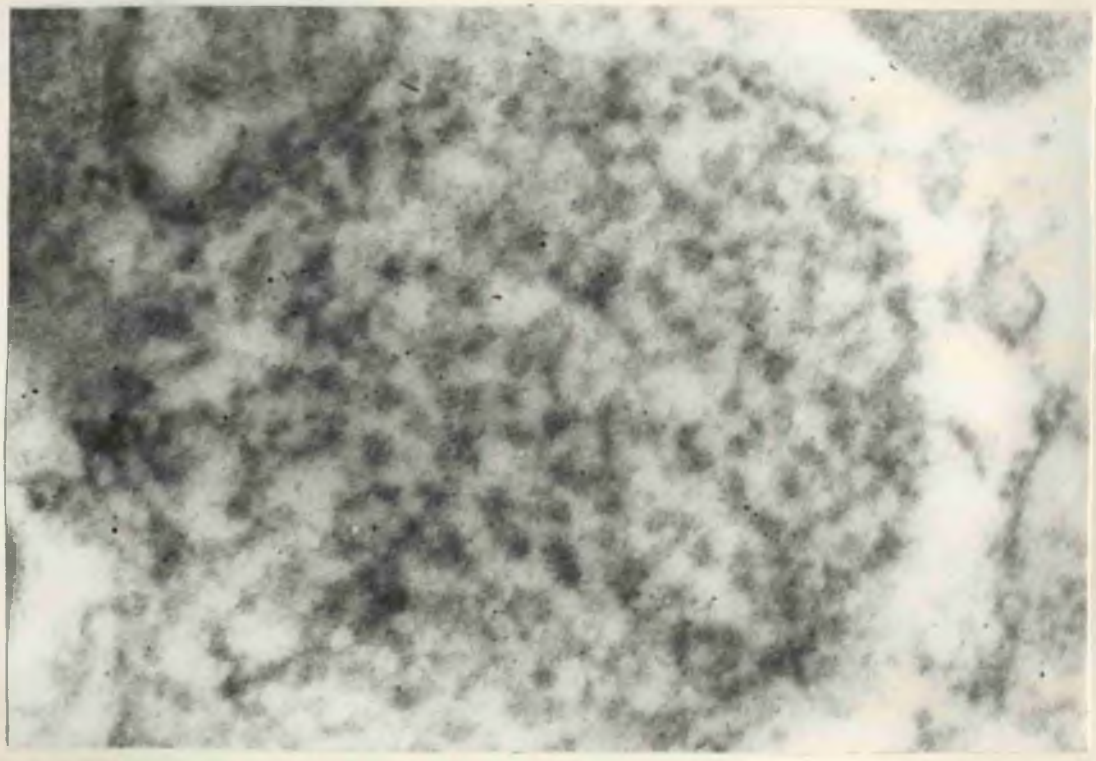


Plate 6

Rat peritoneal mast cell. Disrupted granule stained with uranyl acetate. x 133000.

Plate 7

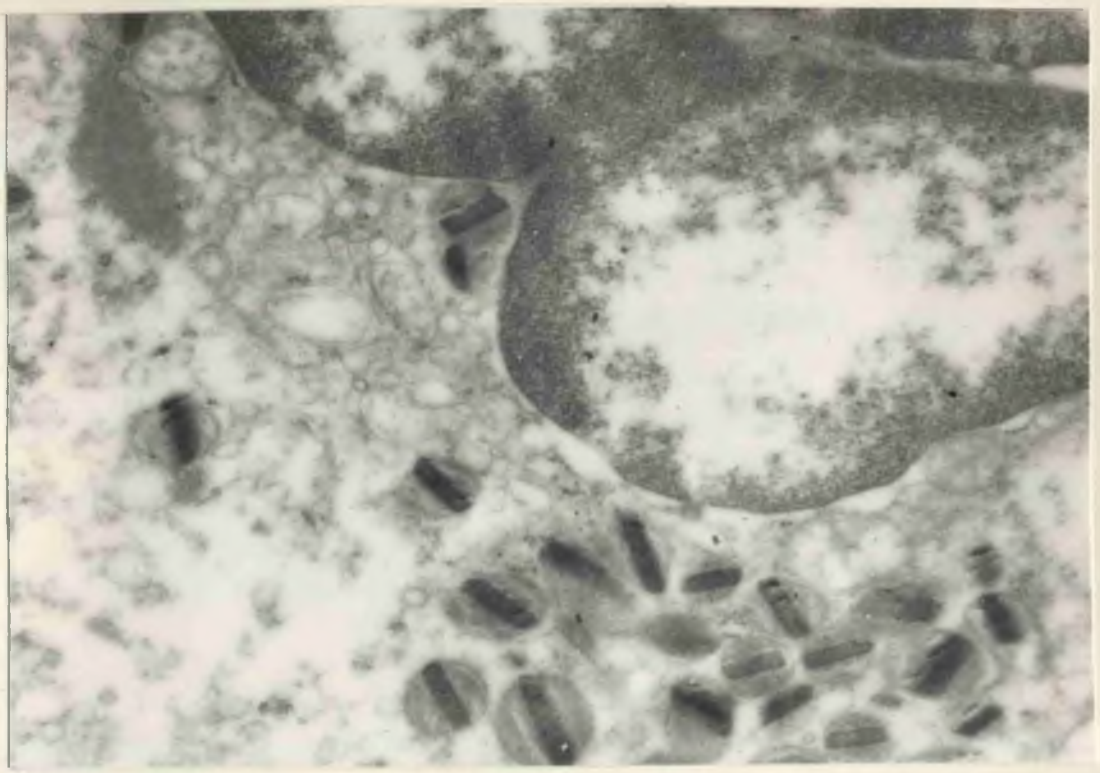


Plate 7

Lymphocyte stained with bismuth nitrate. This plate demonstrates the specificity of the bismuth nitrate stain. x 26000.

BISMUTH NITRATE STAINED MAST CELL GRANULES.

Bismuth staining of mast cells produces an electron density in the granules which, due to their high heparin and hence sulphate content, is very much more intense than that of other components of the cytoplasm. Consequently, staining times which were found optimal for resolution of the granule substructure did not allow clear visualization of the membranes

Plates 8 and 9 show the two types of granule most frequently observed within the mast cells in the course of the present study. The simple granule (Plate 8) possesses a uniform particulate appearance. The compound granule (Plate 9) for the greater part exhibits an identical substructure, but regions of its matrix are also evident which are composed of smaller particles that appear to be stained to a lesser extent, conveying an impression of a more compact nature.

Plate 10 shows a granule in which a great proportion of the matrix components have been released. The stained material appears to form beaded filaments which in some areas coalesce or become interwoven in a loose network. A similar arrangement is evident in the disrupted area (A) of the granule in Plate 11. A reticulum composed of heavily stained bodies connected by beaded filaments, therefore basically similar in nature to that present in the disrupted areas, can be visualize in the intact matrix itself (B, Plate 11). When the disrupted region is examined in greater detail, at a higher magnification (Plate 12) the ultrastructural features of the network become

more evident. The particles in the heavily stained bodies show a considerable variation in size, whereas the diameter of the particles arranged in the filaments averages 35Å.

Plate 8

Rat peritoneal mast cell stained with bismuth nitrate. Simple granule showing a homogeneous particulate matrix. x 63000.

Plate 9

Rat peritoneal mast cell stained with bismuth nitrate. Compound granule in which an area of low electron density is evident. x 59000.

Plate 8 Plate 9

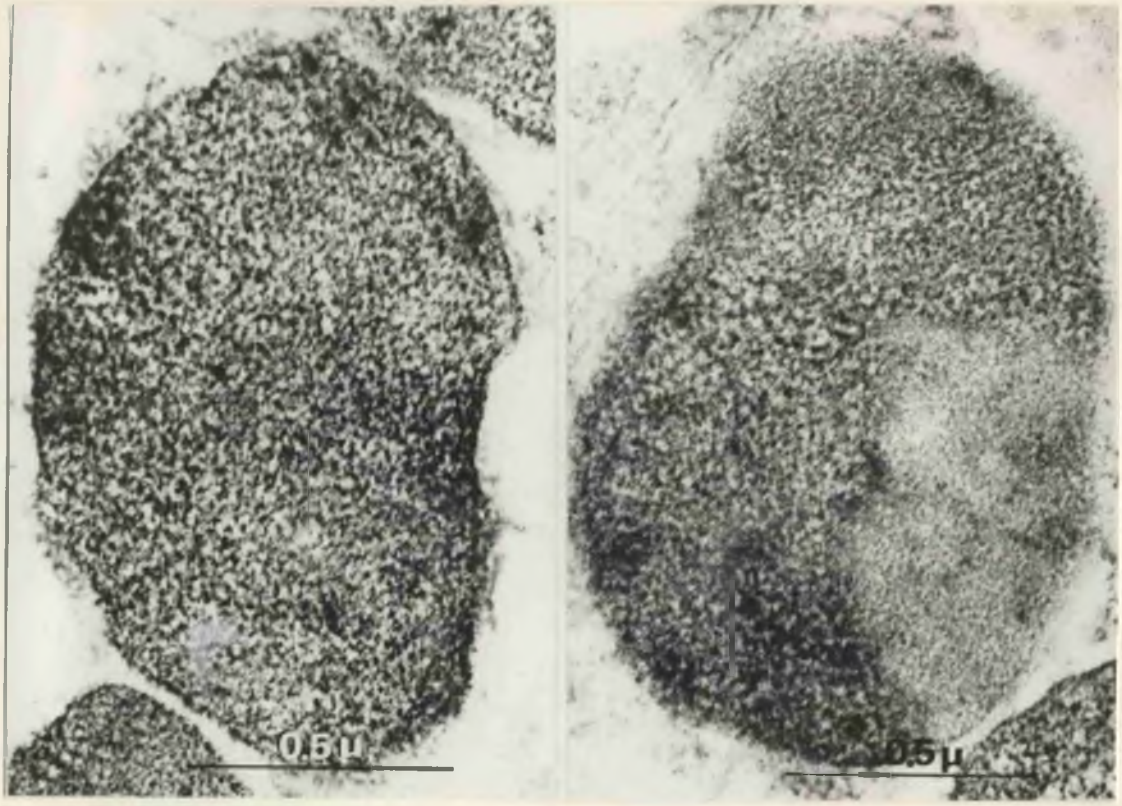


Plate 8

Rat peritoneal mast cell stained with bismuth nitrate. Simple granule showing a homogeneous particulate matrix. x 63000.

Plate 9

Rat peritoneal mast cell stained with bismuth nitrate. Compound granule in which an area of low electron density is evident. x 59000.

Plate 10

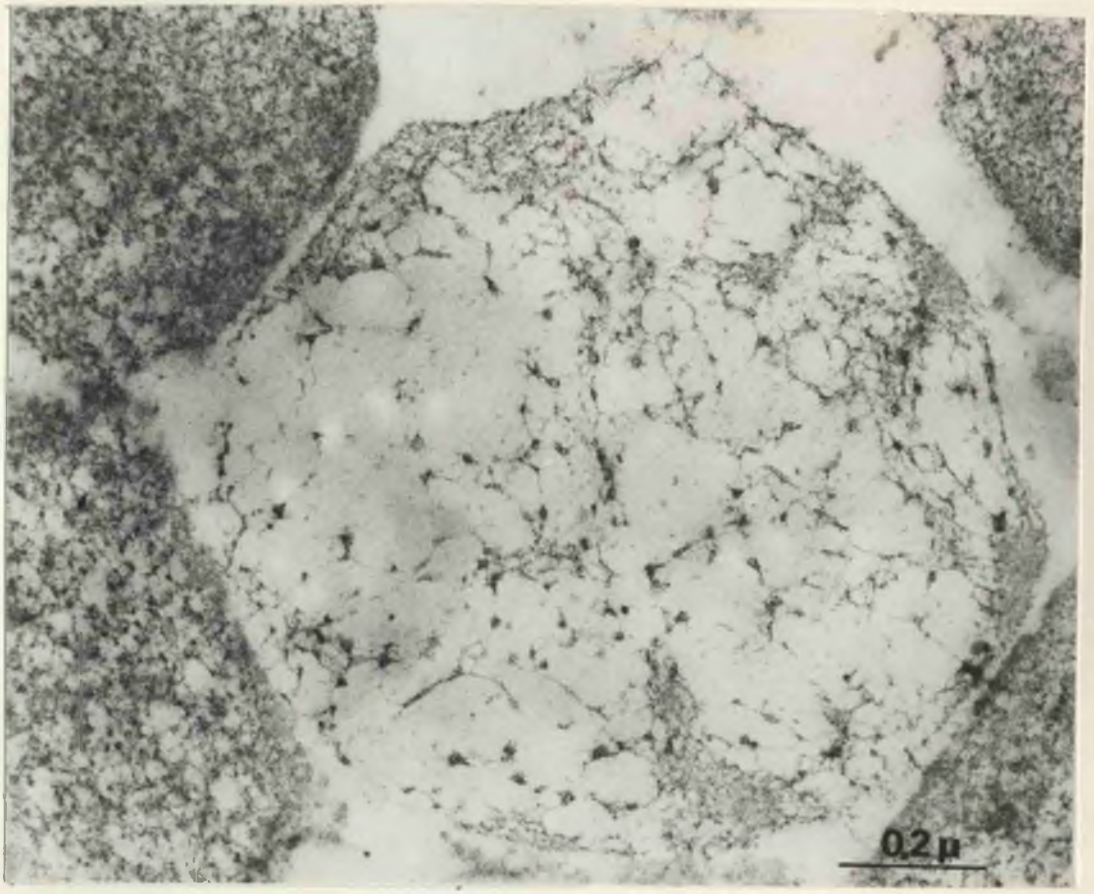


Plate 10

Rat peritoneal mast cell stained with bismuth nitrate. Granule in which a large proportion of the matrix has been released. Stained particles are arranged in filaments which tend to form a network in discrete areas. x 87500.

Plate 11

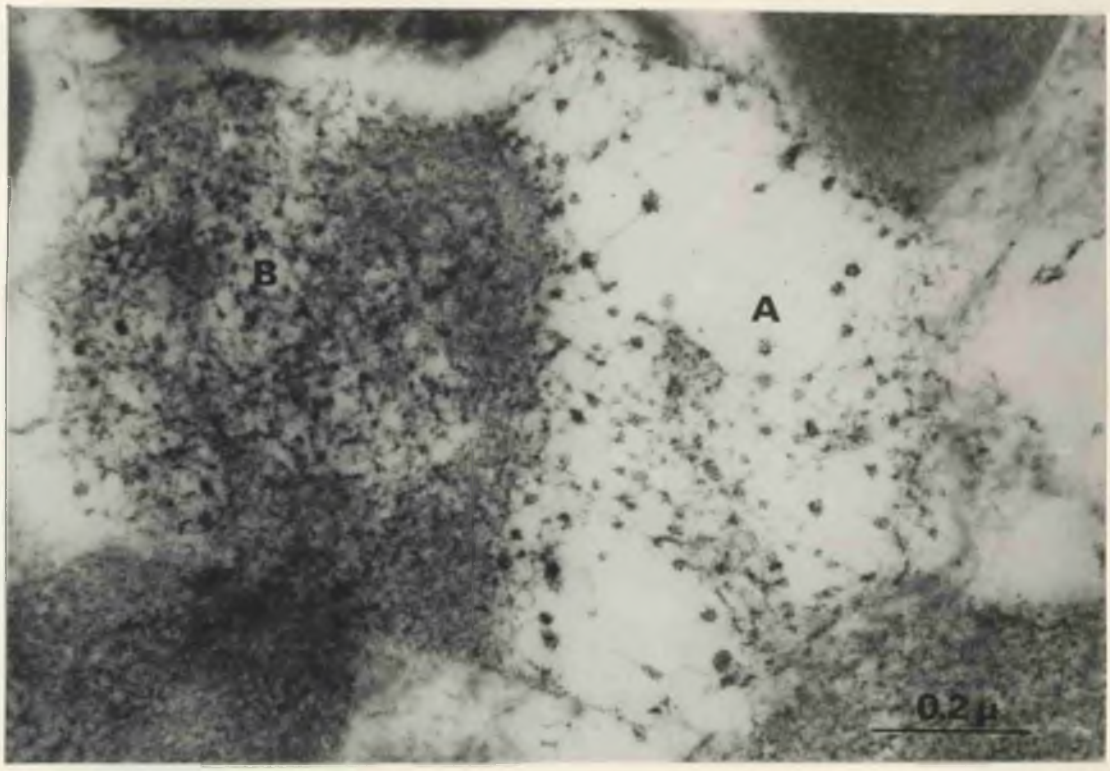


Plate 11

Rat peritoneal mast cell stained with bismuth nitrate. Granule showing a disrupted area (A) and a portion of still intact matrix (B). x 92500.

Plate 12

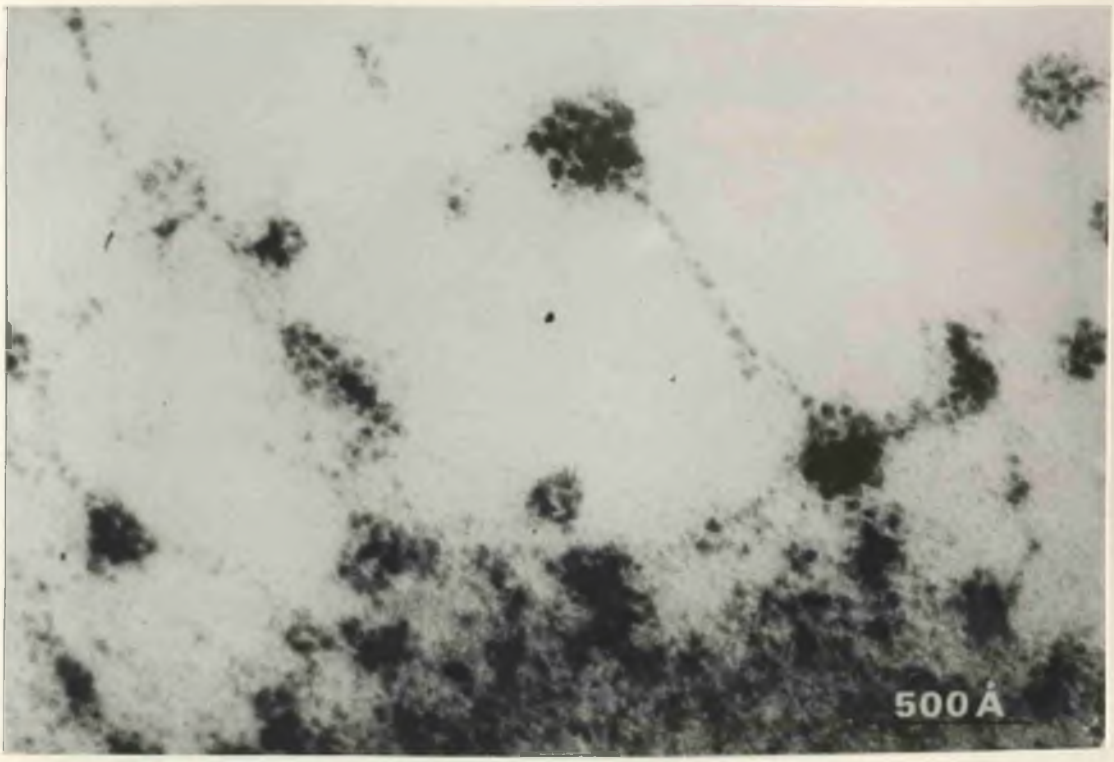


Plate 12

Enlargement of an area from A in Plate 11. x 420000.

DISCUSSION

The heparin content of the average mast cell has been reported to be in the range 30-40 uug (Benditt, Arase & Roeper, 1956). If a heparin-protein complex is to be isolated in appreciable quantities, therefore, it is essential to extract a tissue which is rich in mast cells and, from the research of Riley (1959), it appears that ox liver capsule is such a tissue.

The procedures hitherto devised for the isolation of protein-polysaccharide complexes have employed high-speed homogenisation in the presence of water or salt solutions (Schatton & Schubert, 1954; Malawista & Schubert, 1958; Partridge, Davis & Adair, 1961). These methods rely for their effectiveness on a large amount of free protein-polysaccharide in the tissues. It is known that heparin has the property of binding to collagen (Serafini-Fracassini, 1967), lipids (Bernfield, Donahue & Berkowitz, 1957) and other materials (Korn, 1959). Since the major component of ox liver capsule is collagen, high-speed homogenisation in water or salt solutions alone would result in the release of the heparin from the mast cells followed by its immediate association with the collagen fibres. In this study, this was prevented by the use of cetylpyridinium chloride, which effectively blocks the sulphate groups on the polysaccharide without decreasing its solubility, if in the presence of 2M-potassium chloride (Scott, 1960). Moreover, cetylpyridinium chloride is a well known

deproteinizing agent and allows fractionation of heparin from other mucopolysaccharides and DNA present in the extraction medium by stepwise dilution of the initial supernatant. The subsequent dissociation and removal of the cetylpyridinium ions involves the use of a chloroform-pentan-1-ol mixture which would remove any residual non-covalently bound protein (Blix & Snellman, 1945).

This isolation procedure does not involve the use of either alkali or enzymes which would cause degradation or modification of both protein and polysaccharide components. There is the possibility, however, that during the initial manipulation of the capsule material some degradation of the heparin-protein complex could occur due to the action of trypsin-and chymotrypsin-like enzymes that are known to be present in mast cells (Benditt & Arase, 1959; Lagunoff & Benditt, 1963), but, since trypsin is known to be inhibited by low concentrations of soaps (Peck, 1942), and the stability of both enzymes is temperature dependent, particularly at neutral pH (Anson & Mirsky, 1934; Eisenberg & Schwert, 1951), it is considered that a combination of a high concentration of detergent and potassium chloride, at a temperature of 50°C which is used in the isolation procedure, would prevent hydrolytic damage.

The isolated material behaves, during ultracentrifugal analysis, as a single peak characterized by a sedimentation coefficient of 1.85S at a concentration of 0.95% (w/v). It is considered, however, that the ultracentrifugation pattern

alone does not exclude the possibility of contamination by either non-covalently bound protein or glycopeptide-like materials (Braswell, 1968). However, the preparation examined, once freed from cetylpyridinium chloride, behaves as a single component during ethanol fractionation and requires high concentrations of both ethanol and potassium acetate before precipitation. It is considered that in such an environment it is highly unlikely that any protein or peptide not covalently bound to heparin would not be removed. Further, the results of chromatography for neutral sugars show that only xylose and galactose are present in conformity with the accepted linkage region of heparin to protein (Lindahl & Rodén, 1965). In addition, Serafini-Fracassini et al. (1967) have reported that the protein-polysaccharide of bovine nasal cartilage purified by cetylpyridinium chloride precipitation has a lower protein content than material prepared by the method of Gerber et al. (1960). Lindahl (1969) has criticized the use of cetylpyridinium chloride as a purification agent on the grounds that there is a danger of this compound co-precipitating non-covalently bound protein. The fact, however, that cetylpyridinium chloride purification yields a cartilage proteoglycan having the lowest protein content of all the various methods employed appears to contradict these objections. Moreover, Sidera & Hascall (1969) have shown that chondromucoprotein purified using cetylpyridinium chloride has an amino acid composition virtually identical to that of material prepared by the

dissociation method using guanidinium chloride.

The results of polyacrylamide gel electrophoresis show that, although two heparin proteoglycan regions could be identified in gels by their characteristic metachromasia, after staining with toluidine blue, no orthochromatic bands could be observed. Furthermore, when the gels were stained with amido black, no bands of any description could be seen, indicating that the protein moiety of the material acts as an integral part of the complex during gel electrophoresis.

The molecular weights of the complex, as determined by both N-terminal analysis and osmometry, are in close agreement (19500 and 20600 respectively). Moreover, the average molecular weight for the heparin chains (11300), determined from the xylose content of the material, is in good agreement with the values for the complex as a whole, where two heparin chains are considered to be joined by the protein moiety. If protein contamination had occurred, then the most likely component of the mast cell to be involved is the chymotrypsin-like enzyme which has been reported to exist in association with the heparin in the granules (Lagunoff, 1968), but since this enzyme has a molecular weight of 23000 (Pastan & Almqvist, 1966) this would not be compatible with the molecular weight of the isolated material. Furthermore, this enzyme is known to dissociate from the heparin at potassium chloride concentrations in excess of 1.0M. It is considered, therefore, that the protein component (13.2% of the material)

is entirely covalently bound to the polysaccharide.

In view of the overall similarity of the protein moieties of the complexes of heparin and chondroitin 4-sulphate (Serafini-Fracassini, Peters & Floreani, 1967), a comparison of their amino acid composition is of interest. In both cases, proline, glycine and glutamic acid are the most frequent residues. On the other hand, the serine concentration is decreased in the heparin proteoglycan and becomes equimolar with those of threonine and valine. Many of the other amino acids show slight variations between the two complexes, the major differences being the exceptionally low tyrosine content and the appreciable cysteine or cystine content of the heparin-protein complex. The presence of the latter amino acids suggest that the protein moiety of the complex could play some structural role in the organisation of the mast-cell granules.

The recovered polysaccharide constitutes approximately 82% of the complex and contains 18.1% hexosamine 33.9% hexuronic acid and 10.9% sulphur. These values are in close agreement with the analyses of purified heparin fractions reported by Lindahl et al. (1965). It has been generally accepted, for no apparent reason, that heparin gives high colour yields in the carbazole reaction (Dische, 1947; Bitter & Muir, 1962), but Perlin, Mezurek, Jacques & Kavanagh (1968) suggested, from the results of proton-magnetic-resonance studies on commercial heparin preparations, that the polysaccharide may consist of a repeating trisaccharide composed of equimolar amounts of

L-iduronic acid, glucuronic acid and glucosamine. If this is the case, the values reported in Table 3 for hexuronic acid would be absolute and the sum of all analytical values for the complex would be correct. The high sulphur content of the preparation indicates that the polysaccharide undergoes very little, if any, modification during the extraction procedure.

A further proof of the covalent nature of the complex can now be obtained by chemical means, that is by demonstrating that during β-carbonyl elimination the glycosaminoglycan is cleaved from the protein moiety, as shown by the results of chromatography on AE-cellulose. Moreover, a comparison of the amino acid analyses reported in Table 4, columns (A) and (B) reveals that, during β-carbonyl elimination with 0.5M-sodium hydroxide alone, only the threonine content is significantly reduced, by 19.1%. When the material is subjected to β-elimination and hydrogenation in the presence of Adams catalyst (Table 4, column C), many other component amino acids are destroyed to an appreciable extent. In view of the chemical similarity of threonine and serine on the one hand, and glycine, alanine, leucine, isoleucine and valine on the other, it would be expected that each of these two groups, under identical conditions would be degraded in similar proportions. However, in fact, the threonine content is more drastically reduced (33.8%) than that of serine (5.7%), and the destruction of glycine is only 12.9%, whereas that of alanine, leucine, isoleucine and valine averages 28.0%.

Threonine, therefore, suffers a great deal more destruction than would be expected from catalytic hydrogenation alone, whereas glycine shows a relative increase in proportion to its related compounds. Further α-aminobutyric acid was detected in the hydrolysate of the treated material. These results suggest the conversion of threonine into glycine and α-aminobutyric acid, and such a conversion would be in keeping with the β-elimination mechanism for threonine involved in an O-glycosidic linkage (Adams, 1965; Seno, Meyer, Anderson & Hoffman, 1965).

Lindahl & Rodén (1965), on the other hand, from their study of commercial heparin preparations isolated mainly from pig tissues, by using either proteolytic enzymes or alkali, concluded that serine is involved in the heparin-protein linkage. Although our results appear to be completely at variance with these earlier findings, it is important to point out that in the course of both investigations only 30 of the total theoretical number of linkages have been analysed, and also that the complex examined during the present investigation was isolated by a different procedure from a different tissue and animal species.

Kaplan (1967) has studied the linkage region of a heparatin sulphate-protein complex isolated from the urine of patients suffering from mucopolysaccharidosis III. The major residual amino acid in hydrolysates of this material, after digestion with pepsin and trypsin, was serine, indicating

that this amino acid is involved in the linkage region, in conformity with the results of Muir (1958). On the other hand, when the heparan sulphate-protein complex was subjected to B-elimination in 0.5M-KOH, at room temperature, for 44h, there was no significant loss in serine content. Instead, only threonine showed any significant loss in concentration.

While it is conceivable that the results of β-elimination, both in the present investigation and that of Kaplan (1967), may be misleading, it seems strange that identical treatment of the protein-polysaccharide of bovine nasal cartilage results in a decrease in serine concentration (Anderson, Hoffman & Meyer, 1965). Moreover, after β-carbonyl elimination of the heparin proteoglycan in the presence of Adams catalyst, under hydrogen, α-aminobutyric acid was detected in hydrolysates, confirming that elimination had, in fact, occurred at a threonine residue (Adams, 1965). This apparently contradictory behaviour of both complexes can be explained in two ways. Kaplan (1967) has suggested the possibility of serine being an integral part of the glycosaminoglycan chains, in a similar way to sialic acid and methylpentose present in other mucopolysaccharides. This explanation, however, seems unlikely. An alternative, also proposed by Kaplan (1967), is that linkage through serine is the most frequent type, although a smaller number involving threonine are also present. The xylosylserine linkages, however, are alkali-stable due to either a free amino or free carboxyl group on the amino acid. While the results of amino

end-group analysis of the heparin proteoglycan preclude the possibility of a serine residue with a free amino group, it remains possible that the serine residue involved in the linkage of heparin to protein may be at the C-terminal position of the protein moiety.

The tyrosine content of the material increases from 6.3ug per 10mg of material after a normal 24h hydrolysis to 32.7ug per 10mg material after β-elimination alone and 35.2ug per 10mg material after β-elimination and catalytic hydrogenation. It is impossible to say, at present, whether this behaviour is due either to O-substitution of tyrosine in the heparin proteoglycan, or some compound produced during β-elimination which overlaps the tyrosine peak in the amino acid analyser, thereby producing an apparent increase in tyrosine content.

The anticoagulant activity of the complex is appreciably lower than that of commercial heparin preparations of similar sulphur content. However, it is recognized that, in addition to sulphur content, other molecular features such as size and shape of the polysaccharide influence its biological properties (Jensén, Snellman & Sylvén, 1948; Laurent, 1961; Stivala & Liberti, 1967). Since heparin chains, once arranged in a macromolecular complex with protein, presumably change their conformation, it is impossible, at the present time, to correlate the chemical composition of the heparin proteoglycan and its anticoagulant activity.

Electron microscopy of the preparation, after the first alcohol precipitation, stained with bismuth, (Plates 1, 2) reveals that a proportion of the material appears as beaded filaments of indefinite length composed of particles of 35Å average diameter. In a previous electron microscope study on the light fraction of bovine cartilage mucoprotein, using the bismuth staining technique, Serafini-Fracassini & Smith (1966) have reported that the chondroitin 4-sulphate-protein complex macromolecules were visualized as beaded strands of 1200Å average length, consisting of 20-25 particles, 30Å in diameter. These findings were in conformity with the model proposed on physicochemical evidence, by Ma-thews & Lozaityte (1958) consisting of a central protein core, unstained by bismuth under the experimental conditions, carrying laterally extended chondroitin sulphate chains, at regular intervals. It is interesting, therefore, to compare the morphology of the chondroitin 4-sulphate-protein complex and that of the heparin-protein complex shown in Plate 2. Both macromolecules, in fact, appear as beaded filaments composed of particles of similar size. The slightly larger particle diameter of the coiled heparin chains, 35Å average, is probably due to its higher sulphate content, which results in a more intensely stained and better-defined particle. It is therefore, postulated that both macromolecules have a similar arrangement of the protein core and glycosaminoglycan chains.

In the case of the heparin proteoglycan, however, these long beaded filaments represent only a small proportion of the material, the greater part occurring either as short beaded filaments, individual particles or structureless aggregates.

After repeated alcohol fractionation, long filaments were no longer observed, instead, the preparation appeared as short beaded filaments or individual particles, with an average diameter of 35Å (Plate 3, arrows a). It is impossible, at the present time, to decide whether the absence of long filaments in this highly purified preparation reflects depolymerization occurring during the purification procedure, or if the long filaments constitute part of the 40% insoluble fraction, implying polydispersity of the macromolecules present in the extraction medium. It is interesting to note, however, that a similar type of depolymerization of the light fraction of the protein-polysaccharide complex of bovine nasal cartilage can occur along the core of the macromolecule under conditions in which proteolytic degradation cannot be invoked (Serafini-Fracassini & Smith, 1966).

The highly purified heparin preparation, when subjected to column chromatography on agarose, separates into four fractions. The major fraction (F IV) has been extensively chemically and physicochemically characterized.

A comparison of the chemical composition of fraction F IV (Table 6) with that of the highly purified total heparin

proteoglycan extract (Table 3) reveals that both fractions are basically identical, save for the higher xylose content of the former. It is important to point out, however, that during the anthrone reaction with the highly purified total extract some interference was observed (see Results). It is considered, therefore, that the xylose content determined for F IV is more accurate.

The amino acid analyses of both fractions are also basically identical (Tables 2, 5), save for the high glycine and low histidine content of F IV.

Serafini-Fracassini (1968) has proposed a model for the protein-polysaccharide complex of bovine nasal cartilage in which the macromolecular unit is composed of three 33000 molecular weight sub-units, each of which itself consists of a central polypeptide core and two laterally extending glycosaminoglycan chains. Many of these molecular units are linked in an end-to-end arrangement to form the overall macromolecule.

This model is based on the formation, after treatment of the protein-polysaccharide complex with acidic acetone pH 5.0, of two fragments characterised by sedimentation coefficients of 2.3S and 8.1S respectively. Amino-end group analysis of the acidic acetone treated material reveals the presence of four N-terminal amino acids: aspartic acid, valine, leucine and isoleucine in molar ratios of 1:1:0.7:0.3. The xylose

content of the material is compatible with the presence of two polysaccharide chains per peptide core. The presence of an appreciable amount of keretan sulphate in the 8.1S unit and a lower concentration in the 2.3S unit suggests a possible role for this glycosaminoglycan in cross-linking the sub-units.

In view of the overall similarity of the chondroitin 4-sulphate complex of bovine nasal cartilage and the heparin-protein complex of ox liver capsule on both morphological and chemical grounds, it is interesting to examine this proposed model with respect to the molecular structure of the heparin proteoglycan.

In an electron microscope study of the light fraction of cartilage mucoprotein (Serafini-Fracassini & Smith, 1966), using the bismuth staining method, the protein-polysaccharide molecules were visualized as beaded strands of 1200Å average length, consisting of particles 30Å in diameter. However shorter segments of the material were also observed indicating that fragmentation of the macromolecules can occur under conditions in which proteolytic degradation is impossible. A similar situation is apparent in samples of the heparin proteoglycan as previously described.

The heparin proteoglycan separates on agarose into four fractions, the majority of material being in a single peak characterized by a Stokes radius of approximately 32.5Å, although an appreciable amount of material is present in a

fraction of 94Å stokes radius and two fractions containing small amounts of material of considerably higher molecular weight were also detected. Bovine chondromucoprotein, after treatment with acid acetone, and separation on agarose, produces a similar elution pattern, the fraction corresponding to the 2.3S sub-unit having a Stokes radius of 47.5Å and the 8.1S fraction is eluted at a Stokes radius of 135Å (Serafini-Fracassini & Stimson, 1969). The number average molecular weight of the heparin proteoglycan is approximately 20000, when determined by osmometry and amino-end group analysis. On the other hand, a number average molecular weight of 11000 is derived for the heparin chain from its xylose content. These figures are in agreement with the proposal that the heparin proteoglycan macromolecules, as visualized by electron microscopy, both in extracted material (Plate 2) and within the mast cell granules (Plate 12), are composed of sub-units, each of which itself consists of a central polypeptide core carrying two laterally extending heparin chains. This proposal is in agreement with the model reported by Serafini-Fracassini (1968) for the protein-polysaccharide complex of bovine nasal cartilage. It is impossible to say at the present time, whether the arrangement of these sub-units forming the large macromolecules is identical to that proposed for the chondroitin 4-sulphate-protein complex, but a peak corresponding to a trimer was identified (F III), characterized by a Stokes radius of approximately 94Å.

Although amino-end group analysis of the heparin proteoglycan, unlike that of the chondroitin 4-sulphate-protein complex, yields no precise molar relationship between the various residues, it must be remembered that the chondroitin 4-sulphate complex is isolated as a macromolecule and subsequently reduced to its constituent sub-units, thereby producing a homogeneous mixture of the various molecular species present in the macromolecule. On the other hand, it appears that the majority of the heparin proteoglycan macromolecules are extracted in sub-unit form, and the heterogeniety of the N-terminal amino acids may merely reflect losses at the isolation stage.

The appreciable cystine or cysteine content of the heparin-protein complex (2 residues per polypeptide core) indicates their possible involvement in the structure of the heparin proteoglycan macromolecules. Treatment of the isolated material with 2-mercapto-ethanol, on the other hand, does not produce any results which might clarify this situation. The removal of the heparin proteoglycan from the mast cells by 2M-KCl extraction, observed by many authors (Bloom & Haegermark, 1965; Lloyd, Bloom & Balazs, 1967; Lloyd, Bloom, Balazs & Haegermark, 1967) tends to indicate that the heparin proteoglycan macromolecules do not involve disulphide bridges, although it must be pointed out that these authors did not study the molecular characteristics of their extracted material.

The electron microscopic appearance of rat peritoneal mast cell granules stained with uranyl acetate is shown in Plates 4, 5 & 6. Although this method is insufficiently specific to allow the study of the ultrastructure of the mast cell granules with respect to its heparin component, it shows that the general appearance of the cells studied in this thesis is similar to that reported by other investigators using the same stain (Bloom & Haegermark, 1965; Weinstock & Albright, 1967; Kobayasi, Midtgård & Aboe-Hansen, 1968). The lymphocyte, stained with bismuth nitrate in nitric acid pH 1.2, demonstrates the highly specific nature of this reagent.

Examination of the disrupted region of a rat peritoneal mast cell granule (Plate 11) reveals a network of beaded strands connected by large electron dense bodies. At a higher magnification Plate 12 these beaded strands can be seen to be composed of particles of 35Å average diameter. A comparison of these beaded strands with the filaments in Plate 2 reveals that the particles in both materials have identical diameters. Since the staining method is highly specific for components containing acidic groups of low pK, it is considered that the beaded filaments in Plates 2 & 12 represent heparin proteoglycan macromolecules, composed of a protein core with lateral chains of glycosaminoglycan in coiled conformation. The presence of coiled polysaccharide chains in fixed and embedded material is to be expected since it has been shown that the conformation of heparin in solution

is that of a Gaussian coil with a radius of gyration of approximately 34.5Å (Stivala et al., 1968). The reduction in size of the coils is probably due, in the fixed and embedded material, to dehydration occurring during the preparation of the histological specimens, and, in the isolated material, due to the cross-linking of bismuth ions which would tend to produce a more tightly coiled macromolecule.

The appearance of the large electron dense bodies, which are frequently in continuity with the terminal portion of the filament in Plate 12 suggests that they are probably caused by folding of the protein core resulting in adjacent heparin chains becoming entangled. In such a case, the size of the particles is no longer related to individual chain size, but becomes a function of the degree of entanglement. This interpretation is supported by the fact that entanglement probably also occurs in very dilute solutions, as demonstrated by the existence of particles of large diameter in both sample 1 and sample 2 of the isolated heparin proteoglycan. This is the reason why molecular weight determinations were carried out by osmometry and amino-end group analysis, and not by equilibrium sedimentation. The former methods are not affected very much by interference due to entanglement, whereas equilibrium sedimentation gives a weight-average molecular weight which tends to emphasize disproportionately the higher molecular weight species present.

The continuity in particulate appearance from the filaments into the still intact area of the granule (Plate 11) suggests that elongated macromolecules constitute a large proportion of the granule matrix. It is therefore proposed, that the mast cell granule is composed of a network of heparin-protein macromolecules bound, by means of their intense negative changes, with the basic components of the matrix. This interpretation is in line with the work of Uvnäs & Thon (1965) who demonstrated that histamine is released from the mast cell granules by an ion exchange mechanism. Bloom & Haegermark (1965), from their study of induced histamine release from rat peritoneal mast cells, using the compound 48/80, observed that 75% of the heparin remained inside the mast cell after histamine release, indicating that the heparin-histamine binding is electrostatic. Lagunoff (1968) has proved that the chymotrypsin-like enzyme is bound to heparin in an active form, but on the addition of NaCl in excess of 1M, this complex dissociates, this behaviour being indicative of electrostatic interaction.

The compact regions of the matrix in the compound granule (Plate 9) could consist of macromolecules either with a low degree of sulphation or with their negative changes almost completely neutralised by positive groups. Either of these possibilities, in fact, would result in a low uptake of

bismuth ions, as revealed by the low electron density of these areas.

SUMMARY.

This thesis reports:

1). A procedure for the isolation and purification of the heparin proteoglycan from ox liver capsule, based on the solubility properties of mucopolysaccharide-cetylpyridinium complexes is reported. The yield of the heparin proteoglycan averages 35 mg/100g of dry ox liver capsule.

2). The chemical analysis of the heparin proteoglycan of ox liver capsule is reported. The results of analyses on the polysaccharide show good agreement with values previously published for purified heparin fractions. The amino acid analysis shows several similarities to that of bovine chondromucoprotein. The results of β-carbonyl elimination, either with or without catalytic hydrogenation, and column chromatography after β-elimination, show that the polysaccharide is covalently bound to protein, probably through a xylosylthreonine link.

3). Column chromatography on agarose reveals that the heparin proteoglycan separates into four fractions. Chemical analysis and amino acid analysis on the major fraction shows that it is basically identical to untreated material. Amino-end group analysis reveals the presence of four N-terminal amino acid residues.

4). The major fraction of heparin proteoglycan obtained

after column chromatography is characterised by a Stokes radius of approx. 32.5Å. A number-average molecular weight of 20000 is obtained for this fraction from the results of osmometry and amino-end group analysis. A number-average molecular weight of approx. 11000 is reported for a heparin side chain, based on its xylose content.

5). The heparin proteoglycan, isolated from ox liver capsule, has been studied by electron microscopy after staining with bismuth nitrate. The macromolecules appear as beaded filaments composed of particles of 35Å average diameter.

6). The substructure of the mast cell granules has been examined, using the same staining technique. The matrix of the intact organelle is heavily stained and exhibits a homogeneous particulate appearance. When the granule disrupts, the matrix becomes less compact, revealing a network of fine beaded filaments. The average diameter of the particles arranged in these filaments is 35Å.

7). It is proposed that the mast cell granule is composed of a random network of elongated heparin proteoglycan macromolecules around which the other components of the matrix are arranged. It is further proposed that the macromolecules themselves consist of subunits of 20000 molecular weight, each of which is composed of a

polypeptide core and laterally extending heparin chains. Many of these subunits are joined in an end-to-end arrangement to form the macromolecules.

APPENDIX.

ISOLATION OF THE HEPARIN PROTEOGLYCAN FROM OX LIVER CAPSULE.

During the preparation of this thesis, a paper has been published critising the isolation method described in the present investigation. Lindahl (1970) has attempted to isolate the heparin proteoglycan of bovine liver capsule by a procedure which involves extraction with 2M-KCl, at low temperature, and subsequent purification with cetylpyridinium chloride, as follows:-

100g of ox liver capsule, homogenised by passage through a bacterial press, was suspended in 200ml water and gently stirred for 30min at 0°C. After centrifugation, the extraction was repeated once with water and three times with 2M-KCl, at 4°C. Samples of each extract were tested with cetylpyridinium chloride and only the initial 2M-KCl extract was found to contain any significant polysaccharide content. This KCl extract was clarified by filtration through celite, and precipitated from 0.8M-KCl, containing 0.025M-tris buffer pH 9.0, by cetylpyridinium chloride, the mixture being allowed to stand at 30°C for 30min to allow precipitation. The precipitate was then redissolved in 2M-KCl and reprecipitated by adjustment of the KCl concentration to 0.8M. This second precipitate was suspended in 2M-NaCl in 10% ethanol (v/v) at 30°C, which removed the cetylpyridinium chloride. The polysaccharide fraction was finally precipitated by the addition

of a further 3 volumes of ethanol, collected by centrifugation, washed with ethanol, and dissolved in 5ml of water. This fraction was then subjected to digestion with hyaluronidase for 17h. After digestion, the pH was adjusted to 9.0 and the remaining mucopolysaccharide precipitated with cetylpyridinium chloride and recovered as its sodium salt as previously described. The material was then converted to its calcium salt and fractionated in ethanol by the method of Meyer, Davidson, Linker & Hoffman (1956). The fraction which was precipitated from 50% ethanol was collected, converted into its sodium salt and freeze-dried.

Analysis of this fraction revealed a uronic acid content of 25.8% and a hexosamine content of 20.2% (these values were not corrected for moisture). Glucosamine was reported to constitute 98% of the total hexosamine and a sulphur content of 2.2 moles per mole of hexosamine was obtained. The weight-average molecular weight of the material, as determined by equilibrium ultracentrifugation, was 7400 and its protein content was 3%.

If this material is, in fact, the true native heparin proteoglycan, and our material, as reported by Lindahl (1970) is an artifact due to the non-covalent binding of heparin to a protein contaminant, then the results presented in this thesis would be severely undermined. It is, therefore, necessary to examine in detail the results obtained by Lindahl (1970) and compare them with the analysis of the preparation studied in the present investigation.

It is implied, by Lindahl (1970), that the same extraction procedure is employed in both cases. The procedure used in the present investigation, however, involves homogenisation in 2M-KCl, containing 1% (w/v) cetylpyridinium chloride, preheated to 50°C prior to the addition of dried ox liver capsule, thus ensuring the prevention of any proteolytic enzymic activity. Lindahl (1970), on the other hand, pre-extracts ox liver capsule with water followed by 2M-KCl and subsequent purification using cetylpyridinium chloride. While enzymic activity may be prevented, during extraction, by the use of low temperatures, the exceptionally low molecular weight of the final heparin preparation (7400) has required Lindahl (1970) to propose the existence of a polysaccharidase with endoenzyme properties, which he claims has degraded the heparin moiety of the extracted complex. If an enzyme of this type is present in mast cells, then it would appear likely that under the conditions required for its activity, the trypsin-like and chymotrypsin-like enzymes, known to be present within the mast cell granule itself, would probably degrade the protein moiety of the heparin proteoglycan. The proposal that the chymotrypsin-like enzyme is electrostatically bound, in an active form, to the heparin-protein complex within the granules (Lagunoff, 1968) tends to confirm this observation. In addition, the fact that newly-synthesized polysaccharide is rapidly degraded in mastocytoma cells is not entirely relevant since it has already been reported that heparin isolated from

this type of cell contains no covalently bound protein (Korn, 1959). Moreover, the metabolism of tumour cells is known to be considerably different from that of normal cells, making a direct comparison of their chemical components impossible.

The many techniques employed to ascertain the covalent nature of the protein moiety of the protein-polysaccharide studied in the present investigation have already been discussed at length. The removal of non-covalently bound protein contamination, by Lindahl (1970), performed by repeated re-precipitation of the cetylpyridinium derivative by dilution of the 2M-KCl extract to 0.8M-KCl. However, the observation by Lindahl (1970) that non-covalently bound protein contamination was identified even at the final alcohol precipitation stage of the preparation procedure implies that this procedure was insufficient. Removal of cetylpyridinium chloride in the present extraction procedure was effected by shaking with chloroform:pentan-1-ol (5:4, v/v), which is itself an accepted method of removing non-covalently bound protein (Blix & Snellman, 1945), and the aqueous layer was checked for residual cetylpyridinium chloride by the disappearance of its characteristic E_{260} peak. In addition, after dialysis against distilled water, the material was repeatedly fractionated from ethanol containing potassium acetate, which it is considered would remove any residual protein or peptide bound through electrostatic forces.

The use of cetylpyridinium chloride in the initial extraction medium allows the fractionation of the heparin free from other mucopolysaccharides and DNA by stepwise dilution of the initial supernatant to 0.9M-KCl. A galactosamine content of only 3.6%, when expressed as percentage of total hexosamine, obtained for material purified in this manner confirms that there is very little, if any, contamination by dermatan sulphate. Some commercial heparin preparations have been reported to contain 5.6% galactosamine, expressed as percentage of total hexosamine (Radhakrishnamurthy et al., 1968a). Lindahl (1970), on the other hand, by dilution of a 2M-KCl extract to 0.8M-KCl, recovers a fraction which contains 28% galactosamine. This method, therefore, requires subsequent digestion with hyaluronidase to remove the dermatan sulphate, the galactosamine content after this treatment being reduced to 2% of the total hexosamine.

Amino acid analysis of the heparin proteoglycan isolated by Lindahl (1970) is reported as residues per mucopolysaccharide chain, and only the most abundant amino acids are tabulated. After alkaline treatment in 0.5M-NaOH for 20h at $4^{o}C$, amino acid analysis reveals, in agreement with similar experiments carried out in the present investigation, that the serine content is relatively increased, indicating that the xylosylserine linkage may be at the C-terminal position of the polypeptide. However, whereas after alkaline treatment of the heparin proteoglycan in the present work the serine concentratio

was in fact reduced, but relatively less so than the other amino acids, Lindahl (1970) reports not only a relative increase in its content, but an actual increase in its total concentration compared with untreated material. Moreover, it would appear that in the material subjected to alkaline treatment there is an increase in total protein content compared with untreated material (!).

When heparin is extracted from ox liver capsule, after digestion with papain (Lindahl, 1970), the overall protein content is, as expected, considerably reduced. However, although the serine concentration in this material is relatively increased compared with material isolated under mild conditions, the overall amino acid pattern is basically identical. It is interesting, therefore, to examine the expected action of papain digestion on the heparin-protein complex isolated by Lindahl (1970). It is suggested that this complex is composed of a short polypeptide core to which a single heparin chain is attached. The results of alkaline treatment indicate that the heparin-protein link is probably through xylosylserine, the serine residue being at the C-terminal position of the polypeptide. Papain digestion of a molecule of this type, therefore, would be expected to cause a considerable reduction in its total protein content, and a relative increase in the concentrations of the amino acids nearest to the linkage point, that is a drastic change in the

overall amino acid pattern.

Neutral sugar analysis of the material isolated by Lindahl (1970) reveals a xylose:heparin molar ratio of 0.12, based on a heparin chain consisting of 12 disaccharide units, each of which has a molecular weight of 615. While it is conceivable that heparin may be bound to protein through linkages other than those involving xylose, if it is assumed that only those heparin molecules which contain a xylose molecule are covalently bound to protein, then the 3% protein content of this heparin-protein complex is covalently bound to only 12% of the glycosaminoglycan chains. Therefore, that proportion of the material studied by Lindahl (1970), which in fact contains a covalent protein moiety possesses a protein content of 26%.

In the discussion of his paper Lindahl (1970) quotes the results of Lloyd, Bloom & Balazs (1967) and Lloyd et al. (1967) who isolated small amounts of an alkali-labile heparin-like material from rat mast cells and correlated these results with electron microscope studies on degranulation of the mast cells. Although these workers isolated insufficient material to determine its chemical characteristics, Lindahl proposes that the discrepancy between the results of his own investigation and this previous study might be ascribed to inherent variations among different types of mast cells. However, the material isolated by Lindahl (1970) was not alkali-labile.

In view of the very low molecular weight of the preparation (7400), considerably lower than that reported for some heparin fractions (Lasker & Stivala, 1966; Stivala et al., 1967; Braswell, 1968), and the poor xylose:heparin molar ratio, it is considered that this preparation represents a heparin-protein complex which has suffered extensive enzymatic degradation. Moreover, it has been established (see Discussion) that the protein moiety of the heparin proteoglycan isolated using the procedure described in this thesis is entirely covalently bound to heparin.

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