Attempted Isolation of a Heparin Proteoglycan from Bovine Liver Capsule

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(1) Polysaccharides were isolated from bovine liver capsule by extraction with 2M-potassium chloride followed by precipitation from 0.8M-potassium chloride with cetylpyridinium chloride. Chondroitin sulphate was eliminated by digestion with hyaluronidase. The yield of heparin was approx. 40% of that obtained after extraction of the papain-digested tissue. (2) The macromolecular properties of the hyaluronidase-digested polysaccharide were studied by gel chromatography on Sephadex G-200 of the intact, as well as of the alkali-degraded, material. The results suggested the presence of single heparin chains in addition to a dermatan sulphate proteoglycan. (3) A purified heparin preparation was analysed for amino acids and neutral sugars. Xylose, galactose and serine were found in amounts corresponding to 0.1, 0.2, and 0.4 residue/polysaccharide chain (mol.wt. 7400), respectively. It is suggested that the isolated material had been degraded by a polysaccharidase with endo-enzyme properties.

The existence of complexes between glycosaminoglycans and proteins was first suggested by Mörner (1889), who isolated a crude chondroitin 4-sulphate-protein complex ('chondromucoprotein') from cartilage. More recently proteoglycans containing chondroitin 6-sulphate (Barrett, 1968), dermatan sulphate (Toole & Lowther, 1968) and keratan sulphate (Barrett, 1968) have been isolated and partially characterized. Extraction of tissues by procedures not involving proteolysis yield proteoglycans apparently consisting of multiple polysaccharide chains in covalent linkage to a single polypeptide (termed 'multichain proteoglycan' below). Isolation procedures including treatment of tissues with proteolytic enzymes generally increase the yield of polysaccharide; however, the proteoglycans obtained are degraded, ultimately to single polysaccharide chains linked to residual peptides (Luscombe & Phelps, 1967).

The carbohydrate-protein linkage regions of the proteoglycan molecules have attracted considerable attention. This applies particularly to the chondroitin 4-sulphate-protein linkage region, the structure of which has been established (Gregory, Laurent & Rodén, 1964; Lindahl & Rodén, 1966; Rodén & Armand, 1966; Rodén & Smith, 1966):

A conspicuous feature of this structure is the lability of the xylose-serine linkage, which is the site of cleavage in alkaline degradation of chondroitin 4-sulphate-protein to single polysaccharide chains (Anderson, Hoffman & Meyer, 1965).

Heparin, although structurally closely related to other connective-tissue polysaccharides, displays certain unique characteristics, one being its intracellular location (Brimacombe & Webber, 1964). Nevertheless, the presence of residual amino acids in commercial heparin preparations strongly suggested the existence of a native heparin proteoglycan (Lindahl, Cifonelli, Lindahl & Rodén, 1965). Further studies established the identity of the chondroitin 4-sulphate-protein and the heparin-protein linkage regions (Lindahl & Rodén, 1965; Lindahl, 1966*a,b*, 1967) thus indicating that any multichain heparin proteoglycan should be susceptible to alkali.

Since the existence of a native heparin proteoglycan seemed to be amply verified, attempts were made to isolate this material on a preparative scale from bovine liver capsule. While this work was in progress the results of an identical project were published (Serafini-Fracassini, Durward & Floreani, 1969). However, these latter results are not confirmed by the present study.

MATERIALS AND METHODS

Materials. Bovine liver capsules were removed from the livers within 30 min after the death of the animals and were stored at -20°C after a quick rinse with cold water.

Heparin (prepared from pig intestinal mucosa; anti-coagulant activity, 140 U.S.P. units/mg) purchased from Wilson Laboratories, Chicago, Ill., U.S.A. was purified by repeated precipitation with CPC* from 1.2M-NaCl essentially as described by Lindahl *et al.* (1965). A sample of dermatan sulphate from pig skin was a generous gift from Dr L. Rodén, Departments of Pediatrics and Biochemistry, University of Chicago, Chicago, Ill., U.S.A. Samples of chondroitin 4-sulphate and hyaluronic acid were kindly supplied by Dr E. Wessler of this Institute.

Heparinase (EC 3.2.1.19) isolated from *Flavobacterium heparinum* was generously given by Dr A. Linker, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A. A highly purified preparation of testicular hyaluronidase (EC 3.2.1.35; 20000 i.u./mg) was kindly supplied by Dr B. Högborg, AB Leo, Hälsingborg, Sweden; this material was stated to be free of proteolytic activity. Papain (EC 3.4.4.10; twice crystallized, 29.3 mg of protein/ml of suspension) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Analytical methods. Analyses of total hexosamine were performed by a modification (Gardell, 1953) of the Elson-Morgan reaction after hydrolysis in 4M-HCl for 14 h. The glucosamine/galactosamine ratio was determined by g.l.c. as described by Radhakrishnamurthy, Dalferes & Berenson (1966). Results obtained by this method were compared with those obtained with an automatic amino acid analyser. This double determination was carried out with a large number of glycosaminoglycan samples, including three of the fractions shown in Table 1. Results obtained by the two methods invariably differed by less than 4%. Uronic acid was determined as described by Bitter & Muir (1962). Sulphate was determined by the method of Antonopoulos (1962).

Amino acid analyses were performed after hydrolysis *in vacuo* with 6M-HCl at 110°C for 24 h, with a Bio-Cal model BC-200 amino acid analyser.

Neutral sugars were determined as alditol acetates by g.l.c., by a modification of the procedure of Albersheim, Nevins, English & Karr (1967). Samples of heparin corresponding to 1–10 µg of xylose were hydrolysed with 2M-trifluoroacetic acid at 100°C for 4 h. After the addition of 8 µg of inositol as internal standard, hydrolysates were deacidified by evaporation and passed successively through columns (0.4 cm × 1.5 cm) of Dowex 1 (X2; Cl⁻ form) and Dowex 50 W (X8; H⁺ form), both 200–400 mesh. The use of these ion-exchange resins eliminated a number of unidentified peaks from the chromatograms. Sugars were reduced by treatment with 0.4 mg of KBH₄ in 0.2 ml of m-NH₃ at room temperature for 2 h. After removal of excess of KBH₄ as methyl borate (Albersheim *et al.* 1967) the alditols were acetylated by heating at 100°C for 1 h with 0.2 ml of a mixture (1:1, v/v) of acetic anhydride and pyridine in sealed glass tubes. The residue remaining after evaporation of the acetylation mixture was dissolved in 10 µl of chloroform. Samples (1–2 µl) were separated at 190°C in a Perkin-Elmer model 881 gas chromatograph on glass columns (2 m × 2 mm internal diameter) packed with 1.5% (w/w) ethylene glycol succinate and 1.5% (w/w) silicone oil (XF-1150) on Gas-Chrom P (100–120 mesh). Nitrogen was used as carrier gas, at a flow rate of approx. 30 ml/min. Peak areas were measured with a Technicon

model AAG integrator-calculator. A hydrolysis curve for commercial heparin showed that the hydrolytic conditions chosen resulted in optimum liberation of galactose, whereas the yields of xylose were increased by approx. 10% when the time of hydrolysis was decreased to 2 h. Hydrolysis for 4 h of heparin samples containing known amounts of added xylose and galactose destroyed 15 and 10% of the sugars, respectively. No corrections for destruction during hydrolysis were applied.

Neutral monosaccharides were subjected to paper chromatography in butanol-ethanol-water (10:3:5, by vol.) after hydrolysis of polysaccharides with 2M-trifluoroacetic acid at 100°C for 4 h. Samples were deionized as described by Lindahl & Rodén (1965). Spots were detected by staining with a silver dip reagent (Smith, 1960).

Electrophoresis of polysaccharides was carried out on strips of cellulose acetate in 0.1 M-barium acetate (2.7 V/cm for 6 h) by the method of Wessler (1968). The electrophoretic mobility in this medium depends primarily on the structure of the polysaccharide backbone, whereas variations in sulphate content are of less importance. Before electrophoresis proteoglycans were converted into single polysaccharide chains by treatment with alkali, as described below.

Molecular weights of heparin preparations were determined by equilibrium ultracentrifugation as described by Yphantis (1964) at a concentration of 1.2 mg/ml in m-NaCl. A 12 mm 4° double-sector cell was used at 31410 rev./min and 20°C. The centrifugations were run for about 16 h. The value for partial specific volume used was 0.47 (Lasker & Stivala, 1966).

Extraction and fractionation of polysaccharides

A flow diagram of the preparation procedure is shown in Scheme 1. Frozen liver capsules were homogenized by passage through a bacteria press (Edebo, 1960); this procedure was repeated three times. A sample (100 g) of homogenized material was suspended in 200 ml of water at 0°C and extracted with gentle stirring for 30 min. After centrifugation the extraction was repeated once more with water and three times with 200 ml of 2M-KCl. Care was taken during the entire extraction procedure to keep the temperature below 4°C. Samples of each extract were made 0.8M with respect to KCl and a few drops of 1% (w/v) CPC in 0.8M-KCl were added. Judging from the resulting turbidity significant amounts of polysaccharides were present only in the first KCl extract, which was therefore retained for further fractionation.

The KCl extract, clarified by filtration through Celite, was mixed with an equal volume of 0.05M-tris buffer, pH 9.0, and 0.5 vol. of 1% CPC. The mixture, 0.8M with respect to KCl, was kept in a water bath at 30°C for 30 min and the resulting precipitate, collected by centrifugation, was dissolved in 50 ml of 2M-KCl at 30°C. (On numerous occasions, not mentioned specifically in the text, centrifugation did not effect quantitative sedimentation of CPC-precipitable material. In such instances, the solutions were clarified by filtration through Celite, and polysaccharides were recovered from the Celite by thorough extraction with 2M-KCl.) Insoluble material, probably denatured protein, was removed by filtration through a pad of Celite, which was then thoroughly

* Abbreviation: CPC, cetylpyridinium chloride.

Scheme 1. Flow diagram for fractionation of polysaccharides.

extracted with 2M-KCl. The polysaccharides in the combined filtrate and extract (100 ml) were reprecipitated with CPC from 0.8M-KCl, as described above. The precipitate was suspended in 2M-NaCl in aq. 10% (v/v) ethanol at 30°C; again a portion of the material remained insoluble and was removed by passage through Celite. Polysaccharides were precipitated from the clarified solution (75 ml) by the addition of 3 vol. of 96% (v/v) ethanol. The precipitate (fraction LC) was collected by centrifugation, washed with ethanol and finally dissolved in 5 ml of water.

Digestion of fraction LC with hyaluronidase. Fraction LC (5 ml, containing 6.1 mg of uronic acid) was mixed with 5 ml of 0.3M-NaCl-0.1M-tris buffer, pH 7.0, containing 3 mg of hyaluronidase. A drop of toluene was added and the mixture was incubated at 37°C. After 5 h another 3 mg of enzyme was added and digestion was continued for 12 h. The slightly turbid solution was clarified by filtration through a Celite pad, which was subsequently rinsed with 10 ml of 0.3M-NaCl-0.1M-tris buffer, pH 7.0. The filtrate was adjusted to pH 9.0 with M-NaOH, and polysaccharide was precipitated by the addition of 10 ml of 1% CPC. After reprecipitation with CPC from 0.8M-KCl the

hyaluronidase-digested fraction LC was recovered as its sodium salt (fraction LC-hyase) as described above. As in the preceding part of the preparation, CPC-precipitable material collected during recovery of fraction LC-hyase was only partially soluble in 2M-KCl or -NaCl.

Isolation of polysaccharides after proteolysis of tissue. Homogenized liver capsule (20.2 g) was suspended in 50 ml of 0.05M-sodium acetate buffer (pH 5.5)-0.3M-NaCl-0.01M-EDTA-0.01M-cysteine-HCl. After the addition of 2 ml of papain suspension, digestion was allowed to proceed at 65°C for 20 h. The digest was passed through a Celite pad equilibrated with 0.3M-NaCl and was then mixed with an equal volume of 1% CPC in 0.3M-NaCl. The precipitated polysaccharide was reprecipitated with CPC from 0.8M-KCl and finally converted into the sodium salt, yielding fraction LC-papain.

Gel chromatography of fraction LC-hyase. Fractionation of LC-hyase according to molecular size was accomplished by gel chromatography on a column (0.9 cm × 90 cm) of Sephadex G-200. Samples (1 ml) were mixed with equal volumes of 4M-KCl and elution was carried out with 2M-KCl, at a rate of approx. 4 ml/h. Effluent fractions were analysed for uronic acid by the carbazole reaction.

Ethanol fractionation of fraction LC-hyase. Fraction LC-hyase, as the calcium salt, was fractionated with ethanol by the method of Meyer, Davidson, Linker & Hoffman (1956). The precipitated polysaccharides were converted into sodium salts and freeze-dried. A protein-rich material (LC-hyase-protein), which remained insoluble on dissolving freeze-dried LC-hyase in 5% (w/v) calcium acetate-0.5M-acetic acid, was retained for amino acid analysis.

Degradation of polysaccharides. Attempts were made to effect alkaline cleavage of polysaccharide-protein bonds by mixing 0.5 ml of polysaccharide solution with an equal volume of M-NaOH. The mixture was kept at 4°C for 20 h and was then neutralized.

Digestion with papain was performed as described above, after freeze-drying of polysaccharide solutions.

Digestion of polysaccharides with heparinase was carried out as described by Linker & Hovingh (1965). Digestion mixtures were passed through columns of Dowex 50 W (X2; H⁺ form), evaporated to dryness and spotted on Whatman no. 1 paper. Papers were developed with ethyl acetate-acetic acid-water (3:1:1, by vol.) and stained with a silver dip reagent (Smith, 1960).

RESULTS

Isolation and identification of polysaccharides

Yields and analytical results for some of the polysaccharide fractions are shown in Tables 1 and 2. Since the various fractions had been repeatedly precipitated with CPC from 0.8M-potassium chloride the presence of hyaluronic acid could be excluded (Scott, 1960). Thus, the glucosamine contents of fractions LC-papain, LC and LC-hyase must represent heparin or heparan sulphate; the uronic acid/hexosamine molar ratios indicate the absence of keratan sulphate or glucosamine-containing glycoproteins.

Electrophoresis in barium acetate of fraction LC revealed the presence of three components that migrated like heparin, dermatan sulphate and chondroitin sulphate (Fig. 2). The last component was

completely eliminated by digestion with hyaluronidase, as demonstrated by electrophoresis of fraction LC-hyase (Fig. 1).

The results in Table 1 show that digestion with papain drastically increases the yield of polysaccharides from capsule tissue. However, although less than 20% of the total polysaccharide content of

Fig. 1. Electrophoresis of polysaccharides in barium acetate. C-4-S, chondroitin 4-sulphate; Hep, heparin; HA, hyaluronic acid; DS, dermatan sulphate.

Fig. 2. Chromatography on Sephadex G-200 of: (a) fraction LC-hyase (●) and commercial heparin (×); (b) alkali-treated LC-hyase I; (c) alkali-treated LC-hyase II. Effluent fractions were analysed for uronic acid by the carbazole reaction.

Table 1. Analysis of polysaccharide fractions

The glucosamine/galactosamine ratios of fraction LC and its subfraction LC-hyase refer to different preparations of liver-capsule polysaccharides. The remaining subfractions are all derived from the LC preparation shown in the table.

Fraction	Yield (mg of uronic acid/100g of tissue)	Ratio uronic acid/ hexosamine	Glucosamine (% of total hexosamine)
LC-papain	35.9	1.34	34
LC	6.1	1.30	72
LC-hyase	5.6	1.22	75
LC-hyase I	—	—	47
LC-hyase II	—	—	95

fraction LC-papain was recovered in fraction LC [not considering the somewhat differing colour yields of iduronic acid and glucuronic acid in the carbazole reaction (Bitter & Muir, 1962)], the latter fraction accounted for about 40% of the glucosamine and thus, presumably, the heparin content of the papain-digested fraction. Apparently, heparin is extracted with greater ease than the galactosamine-containing polysaccharides. The yield of fraction LC should be evaluated in the light of the difficulty of removing large amounts of denatured protein without concomitant loss of polysaccharide.

Fraction LC-hyase was further fractionated by gel chromatography on Sephadex G-200, yielding LC-hyase I and II (Fig. 2a). Electrophoresis in barium acetate of LC-hyase I, which emerged at the void volume (V_0) of the column, showed the presence of heparin and dermatan sulphate. Distinct spots appeared only when the material had been subjected to alkaline conditions (see the Materials and Methods section) before electrophoresis. Fraction LC-hyase II was retarded relative to a sample of commercial heparin on gel chromatography (Fig. 2a). Electrophoresis showed one major spot that had migrated like heparin, along with a trace of dermatan sulphate. The presence in LC-hyase I of approximately equal amounts of heparin and dermatan sulphate, as suggested by electrophoresis, was confirmed by hexosamine analysis (Table 1), which showed almost equimolar amounts of glucosamine and galactosamine. The glucosamine content of LC-hyase II indicated heparin as the major polysaccharide constituent.

An attempt to achieve complete separation of the heparin and dermatan sulphate of Fraction LC-hyase by ethanol fractionation was unsuccessful. Material precipitated by 25% (v/v) ethanol was mainly heparin, since glucosamine constituted 77% of the total hexosamine content. On reprecipitation of this material (LC-hyase-25% EtOH; Scheme 1) from 18% (v/v) ethanol (LC-hyase-18% EtOH) the corresponding value decreased to 69%. Dermatan sulphate free of heparin was thus not obtained. On the other hand, dermatan sulphate was quantitatively eliminated from fraction LC-hyase by precipitation from 25% ethanol, since the remaining polysaccharide material (LC-hyase-50% EtOH) was practically devoid of galactosamine (Table 2) and showed only heparin on electrophoresis in barium acetate. In addition to these observations fraction LC-hyase-50% EtOH, which accounted for about 40% of the glucosamine content of fraction LC-hyase, was identified as heparin by the following criteria: (a) resistance towards hyaluronidase; (b) high sulphate content (Table 2); (c) susceptibility to heparinase. Paper chromatography of a heparinase digest showed a pattern of low-molecular weight products identical with that obtained with a digest of commercial heparin.

Table 2. *Analysis of fraction LC-hyase-50% EtOH*

Uronic acid (% of material*)	25.8
Hexosamine (% of material*)	20.2
Glucosamine (% of total hexosamine)	98
Sulphate/hexosamine molar ratio	2.2
$\bar{M}_w$	7400

* Not corrected for moisture

Macromolecular properties of polysaccharides

The retarded position of peak II in the gel chromatogram of fraction LC-hyase (Fig. 2a) indicated that the corresponding polysaccharide occurred as single chains and not as a multichain proteoglycan. Accordingly, the elution position of alkali-treated peak II was the same as that of the untreated material (Fig. 2c).

Alkali treatment of peak I, on the other hand, resulted in a marked degradation of the material (Fig. 2b), indicating the breakdown of a multichain proteoglycan to single polysaccharide chains. However, since part of the alkali-degraded material still emerged at the void volume of the column, the possibility remained that only one of the two polysaccharides, heparin and dermatan sulphate, present in LC-hyase I had been affected by the alkaline conditions. To clarify this point the alkali-treated LC-hyase I was separated into a higher- and a lower-molecular-weight portion, LC-hyase Ia and Ib, respectively (Fig. 2b). Fraction LC-hyase Ia consisted of both heparin and dermatan sulphate, as indicated by electrophoresis, whereas the polysaccharide of fraction LC-hyase Ib was almost exclusively dermatan sulphate. Considering the elution behaviour of the single heparin chains (Fig. 2a) the predicted elution position of heparin liberated from a multichain proteoglycan would be that of fraction LC-hyase Ib. The absence of heparin in this fraction therefore strongly indicates that fraction LC-hyase I does not contain any significant amounts of alkali-labile multichain heparin proteoglycan. It is concluded that fraction LC-hyase I consists of a dermatan sulphate multichain proteoglycan along with single heparin chains or heparin-peptides of relatively high molecular weight. The presence of single chains of dermatan sulphate as well cannot be excluded.

Amino acids and neutral sugars in fraction LC-hyase-50% EtOH

The low molecular weight of Fraction LC-hyase-50% EtOH indicates that this material is essentially the equivalent of fraction LC-hyase II. However, since fraction LC-hyase-50% EtOH seemed to contain heparin of somewhat higher purity than fraction LC-hyase II (Tables 1 and 2) the former material was used for amino acid and neutral sugar

Fig. 3. Amino acid and neutral sugar contents of fraction LC-hyase-50% EtOH. Before analysis, samples *a*, *b* and *c* were precipitated with CPC from 0.5M-KCl and recovered as sodium salts. Recoveries were estimated from hexosamine determinations. Amino acid and neutral sugar contents are expressed as residues/polysaccharide chain; for details see the text. Only the eight most abundant amino acids are included. Owing to irregularities of the base-line, lysine and histidine could not be accurately determined by the single-column system used. (*a*), Untreated LC-hyase-50% EtOH; (*b*), treated with alkali; (*c*), digested with papain; (*d*), fraction LC-hyase-protein (results expressed as μ moles/mg of freeze-dried material).

analyses (Fig. 3). Paper chromatograms of deionized hydrolysates showed spots corresponding to xylose and galactose as major components. Before amino acid analysis samples were precipitated with CPC (see the legend to Fig. 3) and the amount of recovered hexosamine was determined. As the weight-average molecular weight (M_w) of fraction LC-hyase-50% EtOH was known (Table 2) the analytical results could be conveniently expressed as the number of residues/polysaccharide chain (Fig. 3). Calculations were based on the assumption that each heparin molecule consisted of 12 repeating disaccharide units (mol. wt. 615, estimated for the sodium salt of a disaccharide with 2.5 molecules of sulphate).

The total amino acid content of fraction LC-hyase-50% EtOH was low, accounting for only about 3% of the dry weight (freeze-dried material). Serine was the predominant amino acid, amounting to approx. 1 residue/2 polysaccharide chains, followed by glycine, aspartic acid and alanine. However, only a minor fraction of the serine residues could be directly bound to xylose, as the xylose/serine and xylose/heparin molar ratios were 0.28 and 0.12, respectively. In contrast, a sample of commercial heparin showed a xylose/serine molar ratio 0.95 when subjected to the same analytical procedures.

After treatment of LC-hyase-50% EtOH with alkali, peptide material apparently remained bound to the polysaccharide (Fig. 3*b*). The total amounts of amino acids/polysaccharide chain, and particularly

those of serine, were even somewhat increased. This latter finding probably reflects the difficulties inherent in determining the amino acid content of samples that are composed largely of carbohydrate constituents (Swann, 1968).

Digestion of LC-hyase-50% EtOH with papain markedly decreased the amounts of polysaccharide-bound amino acids (Fig. 3*c*). More than 50% of each amino acid was lost, except for serine, which decreased by only 37%.

DISCUSSION

The macromolecular properties of native heparin are uncertain. In view of the close analogy between heparin and chondroitin sulphate, both with respect to mode of biosynthesis (Silbert, 1966) and structure of polysaccharide-protein linkage regions (Rodén & Smith, 1966; Lindahl, 1967) the existence of a native multichain heparin proteoglycan seems highly probable. On the other hand, the results of the present study show that extraction of heparin under mild conditions from bovine liver capsule yields single polysaccharide chains or polysaccharide-peptides rather than a multichain proteoglycan.

Approx. 40% of the total available amount of heparin was isolated, and it is therefore difficult at present to assess whether the results apply to the total heparin content of the tissue. However, the low recovery does not necessarily imply any struc-

tural inhomogeneity of native heparin, since losses due to manipulation, incomplete disintegration of tissues etc. are unavoidable. It is concluded that the results obtained apply to at least a major fraction of the total heparin content of the tissue.

The low molecular weight of the isolated polysaccharide might reflect the properties of newly-synthesized heparin; however, the possibility also exists that heparin is indeed synthesized as a high-molecular-weight proteoglycan that is subsequently degraded. Although attempts to evaluate these alternatives may seem speculative at present one observation in favour of the latter possibility should be noted. The analytical results for fraction LC-hyase-50% EtOH indicate the presence of one xylose residue/seven-eight polysaccharide chains (Fig. 3a). This finding, in conjunction with the low amino acid content, suggests that most of the heparin molecules have undergone degradation by a polysaccharidase with endo-enzyme properties. Even though heparin-protein linkages not involving xylose may conceivably exist, the amino acid content would be too low to account for a peptide substituent on each of the polysaccharide chains. This conclusion is in agreement with previous reports on the low concentration of amino acids in heparin preparations isolated both with and without preceding proteolytic treatment (Lindahl *et al.* 1965; Cifonelli & Rodén, 1968). Further, studies based on pulse-labelling *in vivo* of heparin in mouse mastocytoma indicate that the newly-synthesized polysaccharide is rapidly degraded to lower-molecular-weight products (U. Lindahl & S. Ögren, unpublished work).

Fig. 3(a) shows that the amounts of serine in fraction LC-hyase-50% EtOH exceed that of xylose. If the binding of peptide to heparin is presumed to occur exclusively by xylose-serine linkages, this finding implies the presence of comparatively large peptides, which are attached to a limited number of polysaccharide chains. These heparin-peptides probably represent incompletely degraded proteoglycan molecules. The high recovery of amino acids after alkali treatment and reisolation of polysaccharide (Fig. 3b) may indicate that the xylose-linked serine residues are terminal amino acids, and thus resistant to alkali by virtue of having either a free carboxyl or free amino group (Neuberger, Gottschalk & Marshall, 1966). The presence of heparin-peptide linkages not involving xylose and serine cannot, of course, be excluded.

The isolation of polysaccharide was complicated by the presence in tissue extracts of CPC-precipitable protein-rich materials (see the Materials and Methods section) which were not covalently bound to the polysaccharide. After precipitation with CPC these contaminants were apparently at least partly resolubilized in concentrated salt solutions, since they remained in the polysaccharide fraction even through

the later stage of the preparation procedure (fraction LC-hyase-protein). The same phenomenon has also been observed with extracts of other tissues and has been the subject of a separate report (Lindahl, 1969). In view of these observations the possibility cannot be excluded that the peptide present in fraction LC-hyase-50% EtOH is in part a contaminant. However, the contaminant portion is probably small, since the amino acid composition of fraction LC-hyase-50% EtOH (Fig. 3a) differs significantly from that of fraction LC-hyase-protein (Fig. 3d). The latter material (see the Materials and Methods section) is a protein-rich contaminant, practically free of hexosamine, with the typical fractionation characteristics related above. Further, the relatively high content of amino acids, particularly serine, in fraction LC-hyase-50% EtOH after digestion with papain and reisolation of polysaccharide (Fig. 3c) supports the view that most of the peptide material present is bound to the polysaccharide through a covalent linkage to serine.

The results obtained in the present study are apparently at odds with those of Serafini-Fracassini *et al.* (1969), who reported the isolation from bovine liver capsule of a heparin proteoglycan containing approx. 13% of protein. As in the present investigation heparin was isolated as the CP-polysaccharide complex; however, only one precipitation was carried out. Possibly the disparate results reflect the difficulties in removing non-covalently-bound protein from heparin by the CPC precipitation technique.

Evidence for the existence of a native multichain heparin proteoglycan has also been reported by Lloyd, Bloom & Balazs (1967) and Lloyd, Bloom Balazs & Haegermark (1967) who isolated small amounts of an alkali-labile heparin-like material from rat peritoneal mast cells. The discrepancy between their results and those of the present study might conceivably be ascribed to inherent variations among different types of mast cells.

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