Biochem. J. (1975) **145**, 53–62
Printed in Great Britain

Macromolecular Properties and End-Group Analysis of Heparin Isolated from Bovine Liver Capsule

By LEIF JANSSON, SÖREN ÖGREN and ULF LINDAHL
Institute of Medical Chemistry, University of Uppsala, and Department of Medical Chemistry, The Royal Veterinary College, The Biomedical Centre, S-751 23 Uppsala, Sweden

(*Received* 1 *July* 1974)

Glycosaminoglycans were extracted from bovine liver capsule with 4M-guanidinium chloride, resulting in solubilization of approx. 90% of the total uronic acid-containing polysaccharide of the tissue. The extracted polysaccharide was purified and fractionated by anion-exchange chromatography on DEAE-cellulose, density-gradient ultracentrifugation in CsCl and finally gel chromatography on Sepharose 4B. By using these procedures, the two major polysaccharide components, dermatan sulphate and heparin, which constituted 55 and 30% respectively of the total glycosaminoglycan content of the tissue, were separated from each other. Analysis of the macromolecular properties of the two polysaccharides showed that heparin existed exclusively as single polysaccharide chains, whereas dermatan sulphate occurred largely as a proteoglycan (protein content, 74% dry wt.). The purified heparin preparation was subjected to sedimentation-equilibrium ultracentrifugation, indicating a molecular weight of 8800. Analysis for neutral sugars (by g.l.c.) showed 0.1 residue of xylose and 0.2 residue of galactose/polysaccharide chain; serine amounted to 0.3 residue/polysaccharide chain. Reduction of the heparin with NaB^3H_4 resulted in incorporation of ^3H, approximately corresponding to one reducible group/polysaccharide chain. The ^3H-labelled sugar residue was liberated by a combination of acid hydrolysis and deaminative cleavage of the polysaccharide with HNO_2; it was subsequently identified as an aldonic acid by paper electrophoresis. Most of the heparin chains thus contained a uronic acid residue in reducing position. It is suggested that heparin isolated from bovine liver capsule is a degradation product released from larger molecules by an endo-glycuronidase.

Most of the glycosaminoglycans in connective tissue occur as proteoglycans, consisting of several polysaccharide chains covalently bound to a common polypeptide (Lindahl & Rodén, 1972). The carbohydrate components of such proteoglycans have been identified as chondroitin 4-sulphate, chondroitin 6-sulphate, keratan sulphate and dermatan sulphate; also there is evidence to indicate that heparan sulphate may exist in the native state as a similar macromolecule (Jansson & Lindahl, 1970; Kraemer & Smith, 1974; A. Hallén & L. Jansson, unpublished work).

Structural studies on commercially available heparin preparations showed the presence of the polysaccharide–protein linkage region, typical of most proteoglycans (Lindahl & Rodén, 1972), involving a galactosyl-galactosyl-xylose trisaccharide sequence linked to a serine residue. The occurrence of heparin proteoglycans, suggested by this finding, was reported by Serafini-Fracassini *et al.* (1969) and by Lloyd *et al.* (1967*a*,*b*).

Attempts in our laboratory to isolate a native heparin proteoglycan from bovine liver capsule yielded single polysaccharide chains and polysaccharide–peptides only, accounting for about 40% of the total heparin content of the tissue (Lindahl, 1970). It was suggested that part of the isolated material had been subjected to degradation by an endopolysaccharidase. This conclusion was disputed by Serafini-Fracassini *et al.* (1973), who claimed that heparin isolated from bovine liver capsule contained one residue of xylose/polysaccharide chain, and further that a major fraction of the polysaccharide preparation occurred as doublet molecules, consisting of two heparin chains linked to a common polypeptide. Because of these conflicting findings we reinvestigated the problem.

In the present study heparin was isolated from bovine liver capsule by the use of mild but efficient extraction procedures; the yield exceeded 80% of the total heparin content of the tissue. The macromolecular properties of the isolated polysaccharide conformed in all respects to our previous findings (Lindahl, 1970), thus demonstrating that practically all of the heparin in bovine liver capsule occurs as single polysaccharide chains. Further, most of

these molecules were shown to lack the polysaccharide–protein linkage region, instead having a reducible, terminal uronic acid residue. It is therefore proposed that a major portion of the liver-capsule heparin is a degradation product released from a larger molecule by an endo-glycuronidase.

Experimental

Materials

Bovine liver capsules (i.e. the fibrous connective tissue capsule also denoted Glisson's capsule) were removed from the livers within 30min after death of the animals, and were carefully freed of liver tissue. Capsules were homogenized by repeated passage through a bacteria press at −20°C (Edebo, 1960) and were then defatted with acetone–ether (Blix, 1941).

Heparin (stage 14; prepared from pig intestinal mucosa) was purchased from Wilson Laboratories, Chicago, Ill., U.S.A., and was purified by repeated precipitation with cetylpyridinium chloride from 1.2M-NaCl, essentially as described by Lindahl *et al.* (1965). Samples of dermatan sulphate, chondroitin sulphate and hyaluronic acid were kindly supplied by Dr. Å. Wasteson of this Institute.

Papain was crystallized from a crude preparation (type II; Sigma Chemical Co., St. Louis, Mo., U.S.A.) by the procedure of Kimmel & Smith (1954), and was used as a suspension containing approx. 26mg of protein/ml.

L-Gulono-δ-lactone was a product of Fluka AG, Buchs S.G., Switzerland. D-Glucuronic acid was purchased from Sigma Chemical Co. [^{14}C]Glucuronosyl-anhydromannitol was isolated as described by Lindahl *et al.* (1973) from labelled, non-sulphated polysaccharide synthesized with a mastocytoma microsomal fraction. [U-^{14}C]Glucose (360mCi/mmol) and NaB^3H_4 (6.3Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Stock solutions of labelled NaB^3H_4 were prepared by dissolving portions (100mCi) in 0.1ml of 0.1M-NaOH (Koeltzow *et al.*, 1968).

DEAE-cellulose anion exchanger, Whatman type DE-22, was purchased from W. and R. Balston Ltd., Maidstone, Kent, U.K., and was pretreated according to the manufacturer's instructions before use. Sepharose 4B and Sephadex G-100 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Guanidinium chloride was obtained from Fluka AG, and was purified by treatment with activated charcoal [15% (w/v) suspension in saturated guanidinium chloride]. The charcoal was removed by filtration.

Methods

Analytical methods. Uronic acid was determined as described by Bitter & Muir (1962). Glucosamine/galactosamine ratios were determined by rapid high-pressure ion-exchange chromatography of the amino sugar monosaccharides on Aminex A5 (Bio-Rad Laboratories, Richmond, California) as described by Lohmander (1972); polysaccharides were prepared for analysis by hydrolysis in 4M-HCl at 100°C for 14h. Glucosamine residues with *N*-sulphated (or unsubstituted) amino groups were determined quantitatively by the indole reaction, after conversion into anhydromannose units by treatment with HNO_2 under conditions of reaction A as described by Lindahl *et al.* (1973). Amino acid analyses were performed with a Bio-Cal model BC-200 amino acid analyser, after hydrolysis of samples *in vacuo* with 6M-HCl at 110°C for 24h.

Neutral sugars were determined as alditol acetates by g.l.c. Hydrolysis of polysaccharides and conversion of the resulting monosaccharides into the corresponding reduced and acetylated derivatives were done as described previously (Lindahl, 1970). Samples (1–2μl) were separated at 170°C in a Perkin–Elmer model F30 gas chromatograph, on glass columns (1.80m×2mm internal diameter) packed with 3% (w/w) ECNSS-M on Gas Chrom Q (100–120mesh) (Sawardeker *et al.*, 1965). N_2 was used as carrier gas at a flow rate of approx. 30ml/min. Peak areas were measured by planimetry.

Electrophoresis of glycosaminoglycans was carried out on strips of cellulose acetate, in 0.1M-barium acetate, pH6.6 (2.7V/cm for 6h) (Wessler, 1968) or in 0.1M-HCl, pH1.2 (1.9V/cm for 2h) (Wessler, 1971). The electrophoretic mobility in barium acetate depends primarily on the structure of the polysaccharide backbone, whereas variations in sulphate content are of less importance. In dilute HCl the carboxyl groups of glycosaminoglycuronans are undissociated; the migration rate of polysaccharide in this medium therefore depends exclusively on its degree of sulphation. The combined application of the two methods thus permits the identification of individual glycosaminoglycan components in a polysaccharide mixture. Before electrophoresis, proteoglycans were converted into the single-chain state by treatment with 0.5M-NaOH at 4°C for 20h, followed by neutralization and dialysis.

Gel chromatography of glycosaminoglycans was carried out at 4°C, on columns of Sepharose 4B (1cm×95cm) or Sephadex G-100 (1.2cm×90cm). Samples were usually eluted with 1M-NaCl–0.05M-Tris–HCl, pH7.4 (although samples of ^{3}H-labelled heparin were eluted with the same buffer at pH8.0), at a rate of about 4ml/h.

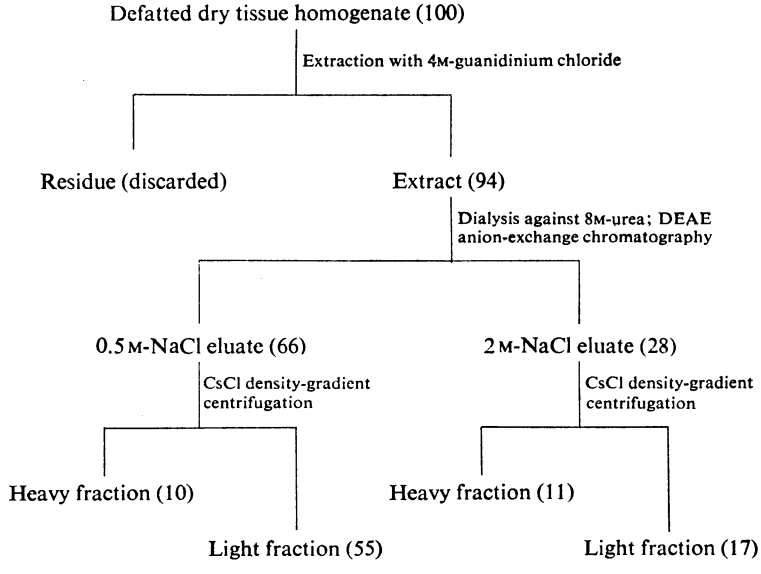
Paper electrophoresis was done with Whatman 3MM paper, at 70V/cm, in 0.08M-pyridine–0.046M-acetic acid, pH5.3, or in 0.06M-pyridine–0.43M-acetic acid, pH3.9. Before electrophoresis lactones of aldonic acids were converted into the free acid form by treatment of the samples with aq. 0.25M-NH_3 for 1h at room temperature. Paper chromatography was

performed with Whatman 3MM papers in ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.). Non-radioactive sugars were stained by a silver-dip procedure (Smith, 1960), whereas ^{14}C-labelled components were located with a Packard model 7201 strip scanner. ^{3}H-labelled components were determined after cutting the paper strips into 1 cm pieces, which were transferred to scintillation vials containing 1 ml of water. About 1 h later, 10 ml of Instagel (Packard Instrument Co., Downers Grove, Ill., U.S.A.) was added; the vials were shaken vigorously and were then counted for radioactivity in either a Packard Tri-Carb 2002 or a Beckman LS 250 liquid-scintillation spectrometer. The counting efficiency for ^{3}H (usually 20–25%) was determined by using $^{3}H_2O$ as internal standard.

Molecular weights of glycosaminoglycans were determined by meniscus-depletion equilibrium ultracentrifugation as described by Chervenka (1970), at a polysaccharide concentration of 2 mg/ml of 1 M-NaCl–0.08 M-Tris–HCl, pH 7.4. A 12 mm 4° capillary-type, synthetic-boundary, double-sector cell was used at 20°C. Heparin and dermatan sulphate fractions were centrifuged for 19 h and for 21 h at 36000 and 22000 rev./min respectively. The values for partial specific volumes used were 0.47 ml/g for heparin (Lasker & Stivala, 1966) and 0.54 ml/g for dermatan sulphate (Luscombe & Phelps, 1967).

Reduction with sodium boro[^{3}H]hydride. A mixture of heparin (109 μg of uronic acid) and [^{14}C]glucose (0.1 μmol; 7×10^{4} c.p.m.) was treated with 0.5 M-$NaB^{3}H_4$ (100 mCi/mmol, as based on the specifications given by the manufacturer) in 75 μl of 0.1 M-sodium borate buffer, pH 8.0. After 3 h at room temperature, the excess of borohydride was destroyed by acidification with acetic acid to pH 5. The solution was passed through a column (1 cm × 1.5 cm) of Dowex 50 (X8; H^+ form) and was followed by a water wash. The pooled effluents were evaporated to dryness four times in the presence of methanol, and the final residue was then dissolved in 50 μl of water. Polysaccharide was precipitated by the addition of 4 vol. of ethanol, and collected by centrifugation; the supernatant containing the reduced [^{14}C]glucose was retained for paper chromatography. After repeated precipitation with ethanol, the polysaccharide (100 μg of uronic acid) was subjected to gel chromatography on Sephadex G-100 (for details, see above), yielding a single peak of radioactivity emerging shortly after the excluded volume of the column. The ^{3}H-containing effluent fractions were pooled, and desalted by passage through a column (3 cm × 30 cm) of Sephadex G-15 eluted with 10% (v/v) ethanol. Degradation of the ^{3}H-labelled polysaccharide, essentially to the monosaccharide level, was achieved by a combination of acid hydrolysis and deamination with HNO_2, as described by Höök *et al.* (1974).

The actual specific radioactivity of the borohydride in the reduction mixture was determined from the



Scheme 1. *Flow diagram of the fractionation procedure*

Values in parentheses refer to the yields of polysaccharide, expressed as per cent of the total uronic acid content of the starting material. For further details see the text.

$^3H/^{14}C$ ratio of the sorbitol formed on reduction of the [^{14}C]glucose internal standard. The conversion of [^{14}C]glucose into [^{14}C,^{3}H]sorbitol was quantitative, as demonstrated by paper chromatography; the $^3H/^{14}C$ ratio of the isolated sorbitol was determined by liquid-scintillation counting as described above.

Isolation of polysaccharide. A flow diagram of the fractionation procedure is shown in Scheme 1. The method used for the extraction and recovery of glycosaminoglycans is based on the procedure described by Antonopoulos *et al.* (1974). Defatted, dry tissue homogenate (60g) was suspended in 1.2litres of 0.05M-sodium acetate buffer, pH5.8, containing 4M-guanidinium chloride, and was extracted under stirring at 4°C for 24h. The extract was clarified by centrifugation at 20000*g* for 60min, dialysed against 8M-urea in 0.05M-sodium acetate buffer, pH5.8, and finally passed through a column (7.5cm × 10cm) of DE-22 anion exchanger, equilibrated with 8M-urea, in the same buffer. After washing with 3 bed-volumes of buffered 8M-urea the retained glycosaminoglycans were eluted in two steps with 3 bed-volumes each of 0.5M-NaCl and 2M-NaCl respectively in buffered 8M-urea. The 0.5M- and 2M-NaCl eluates were dialysed against water and were then freeze-dried.

The two polysaccharide fractions were each separated from contaminating protein and glycoprotein by CsCl density-gradient centrifugation under dissociative conditions (4M-guanidinium chloride in 0.05M-sodium acetate buffer, pH5.8). Centrifugation was carried out at 105000*g* for 48h at 20°C, in a Beckman L2-65B preparative ultracentrifuge (rotor 50.1). The loading density was 1.46g/ml. Fractions of volume about 1.1ml were collected and their densities were determined by use of a calibrated 1000μl pipette.

The total content of glycosaminoglycuronan in the tissue was determined as follows. Homogenized, defatted, dried liver capsule (2.0g) was suspended in 20ml of 0.05M-sodium acetate buffer, pH5.5, containing 0.3M-NaCl, 0.01M-EDTA and 0.01M-cysteine–HCl. After the addition of 0.5ml of papain suspension, digestion was allowed to proceed at 65°C for 20h. The liberated polysaccharide was isolated by precipitation with cetylpyridinium chloride from 0.3M-NaCl. After conversion into the sodium form (Lindahl, 1970), the polysaccharide was quantified by the carbazole reaction.

Results

Fractionation and identification of glycosaminoglycans

The total glycosaminoglycan content of the liver capsule tissue, determined as uronic acid-containing material precipitable with cetylpyridinium chloride from 0.3M-NaCl, corresponded to 0.81mg of uronic acid/g of dry tissue. This value may be somewhat too low, owing to incomplete recovery of hyaluronic acid; however, calculations described in the legend to Fig. 3 indicate that this polysaccharide amounted to at most 10% of the total glycosaminoglycan content of the tissue. By extraction with 4M-guanidinium chloride, polysaccharide corresponding to 0.77mg of uronic acid/g of dry tissue (94% of the total uronic acid) was brought into solution. It may be concluded that at least 80%, and probably more, of the heparin present in the liver capsule was recovered in the extract.

The extracted polysaccharide was fractionated by anion-exchange chromatography on DEAE-cellulose. Approximately two-thirds of the uronic acid-containing material applied to the column was eluted with 0.5M-NaCl, the remainder being recovered in a 2M-NaCl eluate. Electrophoretic analysis of the 0.5M-NaCl and 2M-NaCl eluates showed dermatan sulphate and heparin respectively as the predominant polysaccharide components (Fig. 1). In addition, both fractions contained trace amounts of chondroitin sulphate and hyaluronic acid.

Further fractionation of the 0.5M- and 2M-NaCl eluate fractions was achieved by density-gradient centrifugation in CsCl. The uronic acid-containing

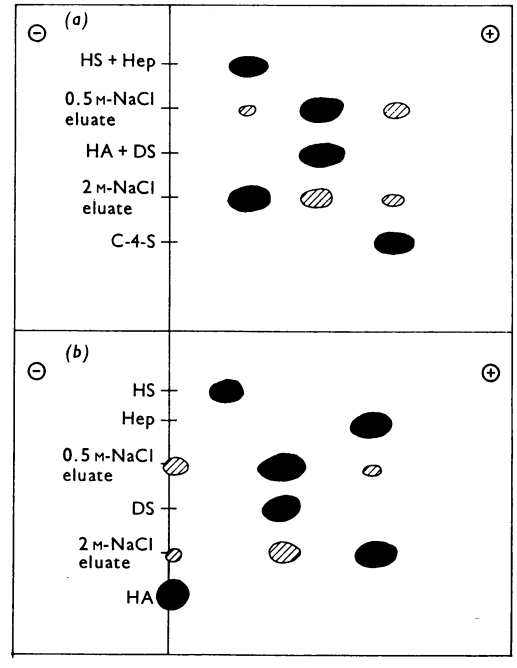


Fig. 1. *Cellulose acetate electrophoresis in* (*a*) 0.1M-*barium acetate and* (*b*) 0.1M-*HCl, of glycosaminoglycans in the* 0.5M-*NaCl and the* 2M-*NaCl eluate fractions*

Standards: C-4-S, chondroitin 4-sulphate; DS, dermatan sulphate; HA, hyaluronic acid; HS, heparan sulphate; Hep, heparin. For details see the text.

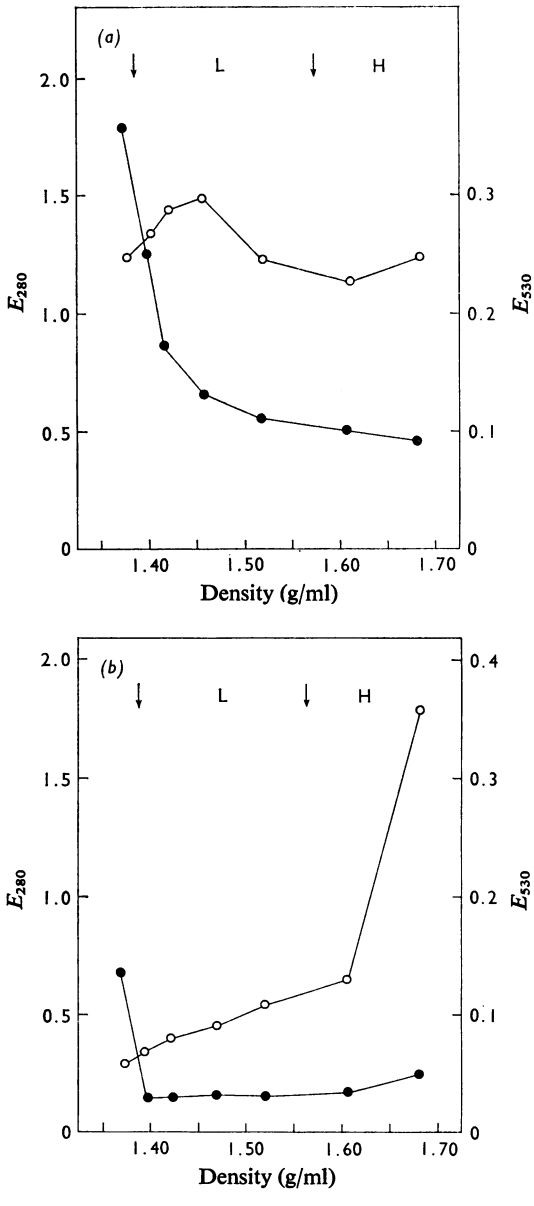


Fig. 2. *Density-gradient centrifugation in CsCl of* (*a*) 0.5M-*NaCl eluate and* (*b*) 2M-*NaCl eluate obtained by anion-exchange chromatography*

Fractions were analysed for uronic acid (○) (carbazole reaction, E_{530}) and for E_{280} (●), and were then combined into 'light' (L) and 'heavy' (H) fractions, as indicated by the arrows. For further details see the text.

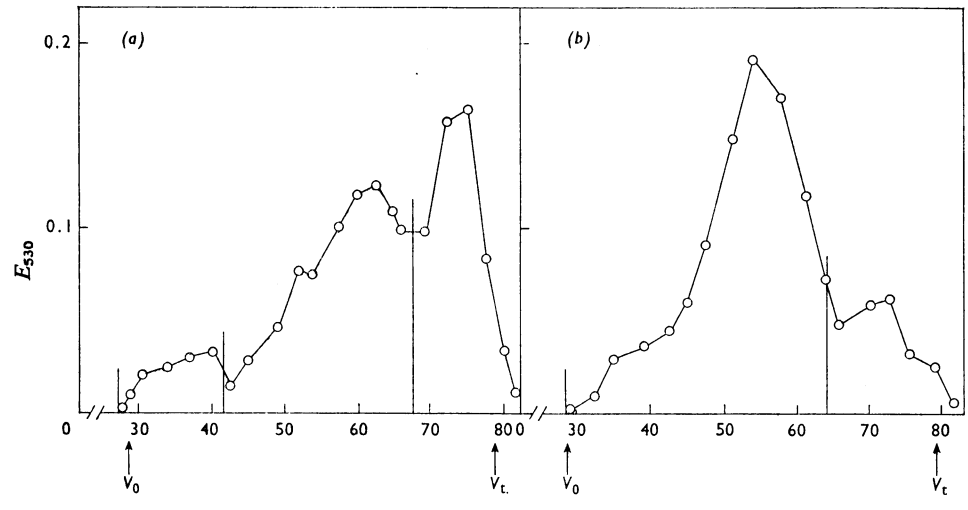
material in the 0.5M-NaCl eluate was distributed over the entire gradient, with a peak between densities 1.40–1.50g/ml (Fig. 2*a*), whereas the material in the 2M-NaCl eluate appeared preferentially in fractions of higher density (Fig. 2*b*). In both separations, contaminating protein or glycoprotein was accumulated in the fractions of lowest density. Gradient fractions were pooled to yield a 'light' and a 'heavy' fraction from each of the 0.5M-NaCl and the 2M-NaCl eluates, as shown in Fig. 2. In the following, the resulting preparations will be designated as 0.5M-heavy, 0.5M-light, 2M-heavy and 2M-light respectively. The distribution of uronic acid-containing material between these fractions is given in Scheme 1.

The fractions obtained by density-gradient centrifugation were separated further by gel chromatography on Sepharose 4B (Figs. 3 and 4). Effluent fractions were combined as indicated in the Figures, and were then analysed for glucosamine/galactosamine ratio and for polysaccharide composition (as determined by cellulose acetate electrophoresis). The 0.5M-heavy and 0.5M-light fractions were both heterogeneous, and showed dermatan sulphate within a wide range of molecular size (Fig. 3). The major portion of the dermatan sulphate occurred in the 0.5M-light fraction, and appeared as a fairly distinct peak with $K_{av.}$ approx. 0.5. The 2M-heavy and 2M-light fractions gave almost identical elution patterns (Fig. 4), with a single, distinct, retarded peak ($K_{av.}$ about 0.9). Heparin was the only polysaccharide detectable on cellulose acetate electrophoresis of the retarded material. In agreement with this finding, glucosamine was the only amino sugar present in significant amounts; further, about 80% of the glucosamine residues were *N*-sulphated (as indicated by the amounts of anhydromannose determined after deamination of the polysaccharide, related to the uronic acid content of the intact polysaccharide; no corrections were applied for the anomalous high colour yields of the indole and the carbazole reactions respectively).

Calculations based on the quantitative data given in Scheme 1 and in Figs. 3 and 4 indicate that heparin and dermatan sulphate constituted about 30% and 55% respectively of the total glycosaminoglycans of the liver capsules. The high-molecular-weight portion of the 0.5M-light fraction (dermatan sulphate; see Fig. 3*b*) and a low-molecular-weight portion of the 2M-heavy fraction (heparin; see Fig. 4*a*) were dialysed, freeze-dried and retained for further analysis.

Macromolecular properties of heparin and dermatan sulphate fractions

Digestion of the isolated dermatan sulphate with papain resulted in considerable degradation, as shown by gel chromatography (Fig. 5), indicating breakdown of a proteoglycan structure to single polysaccharide chains. The molecular weight of such chains (represented by the peak fractions of the degraded



Molar ratio...	1.00	0.73	0.32	0.85	0.88
Electrophoresis pattern...	CS,DS	(Hep), HA, DS, CS	(Hep), DS, HA	DS, (HA)	DS, (HA)

Fig. 3. *Gel chromatography on Sepharose 4B of (a) 0.5 M-heavy fraction and (b) 0.5 M-light fraction*

The amino sugar composition [given as molar ratio of galactosamine/(galactosamine+glucosamine)] and electrophoresis patterns of effluent fractions, pooled as indicated by the vertical lines, are shown below the chromatograms. Polysaccharide components are indicated by the abbreviations defined in the legend to Fig. 1, and represent the combined results of electrophoresis in barium acetate and in HCl respectively (see under 'Analytical methods'). Components occurring in minor amounts appear within parentheses. The maximum amount of hyaluronic acid was calculated by assuming that all glucosamine occurring in the 0.5 M-light and 0.5 M-heavy fractions was a component of this polysaccharide. By relating the hexosamine composition of the pooled subfractions in Figs. 3(*a*) and 3(*b*) to the corresponding total amounts of uronic acid (obtained from the yields given in Scheme 1 and the gel-chromatography patterns in Figs. 3*a* and 3*b*) the amount of hyaluronic acid was found to comprise at most 10% of the total glycosaminoglycan. For further details see the text.

material; see Fig. 5) was estimated by ultracentrifugation (Fig. 6*b*) to be 19800. In contrast the heparin fraction retained the same molecular size during treatment with either papain or alkali (0.5 M-NaOH at 4°C for 20h). The molecular weight of the combined heparin peak fractions (see Fig. 4*a*) was 8800 (Fig. 6*a*).

It is concluded from these results that most or all of the heparin in bovine liver capsule occurs as single polysaccharide chains, whereas a major portion of the dermatan sulphate exists in the tissue in proteoglycan form.

Amino acid and neutral sugar contents of heparin and dermatan sulphate fractions

The results of amino acid and neutral sugar analyses are shown in Table 1. The total amino acid content of the liver capsule heparin (2 M-heavy fraction) was low, amounting to less than 3% of the dry weight, serine and glycine being the most abundant amino acids. By assuming a molecular weight for the heparin of 8800, as determined, the amount of serine corresponded to approximately one residue for every third polysaccharide chain. The xylose content, determined by g.l.c. (Fig. 7), was even lower, about one residue for every eight polysaccharide molecules. A similar low xylose content (0.18 residue/polysaccharide chain) was determined in the 2 M-light heparin fraction.

The dermatan sulphate preparation (high-molecular-weight portion of 0.5 M-light fraction) contained large amounts of protein, accounting for about three-quarters of the dry weight of the material (Table 1). Also the xylose content (determined on papain-digested polysaccharide) was higher than that of the heparin fractions, corresponding to about one residue per dermatan sulphate chain.

According to the current concept of heparin and dermatan sulphate structures, the linkage between polysaccharide and polypeptide is mediated exclusively by the galactosyl-galactosyl-xylosyl-serine sequence (Lindahl & Rodén, 1972). A conventional

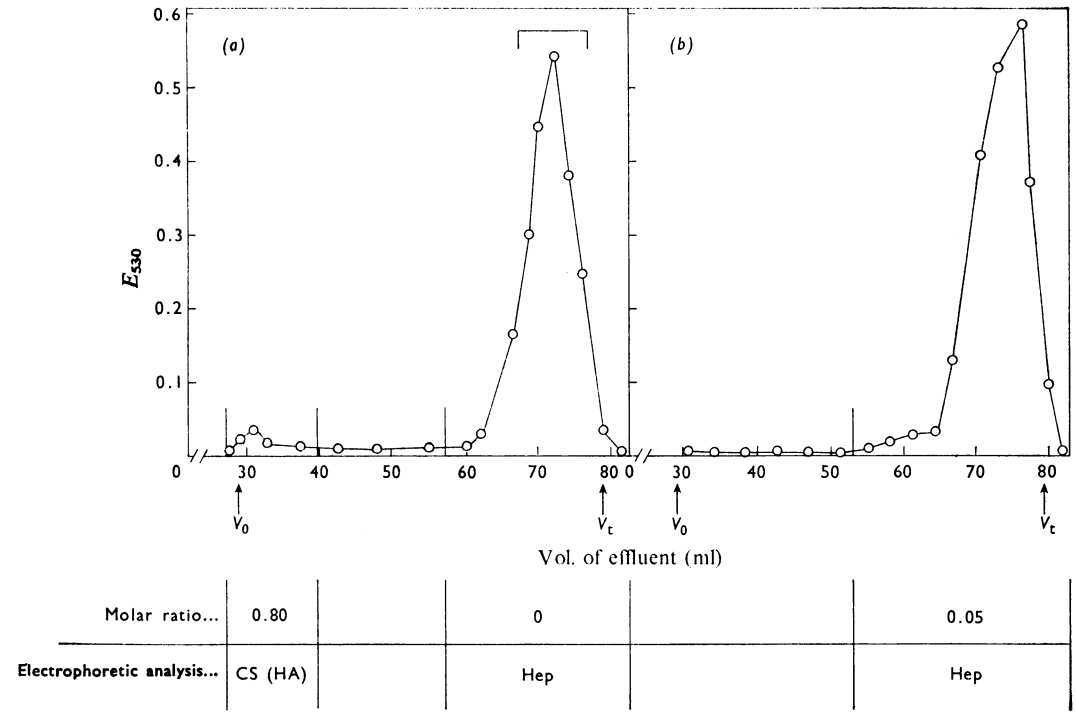


Fig. 4. *Gel chromatography on Sepharose 4B of (a) 2 M-heavy fraction and (b) 2 M-light fraction*

The molar ratio of galactosamine/(galactosamine+glucosamine) and the results of electrophoretic analysis are shown. For further details, see the legend to Fig. 3. The fractions indicated by the horizontal bracket were pooled and subjected to further analysis. For further details see the text.

Table 1. *Amino acid and neutral sugar analyses of heparin and dermatan sulphate fractions*

For details see the text.

	Residues/polysaccharide chain			Total amino acid content (% dry wt.)
	Serine	Xylose	Galactose	
Heparin (mol.wt. 8800)	0.31	0.13	0.23	2.7
Dermatan sulphate (papain-digested single chains; mol.wt. 19800)	1.4	1.2	1.6	4.5*

* The intact dermatan sulphate proteoglycan contained 74% protein.

proteoglycan structure should thus display one xylose residue for each constituent polysaccharide chain, as found for the dermatan sulphate fraction. The relative lack of xylose in the heparin fractions therefore strongly indicates that the majority of these molecules were fragments derived from larger compounds, by cleavage within polysaccharide chains and not by proteolytic scission of a polypeptide core. Further, it may be inferred from the serine/heparin and xylose/heparin molar ratios, that only a minor fraction of the serine residues could be directly bound to xylose; the presence on these xylose units of peptides rather than single amino acid substituents thus appears likely. These conclusions are in excellent agreement with our previous characterization of heparin, obtained from bovine liver capsule by a different, and less efficient, isolation procedure (Lindahl, 1970).

Reducible end-group residue in isolated heparin

Cleavage of a polysaccharide chain would be expected to yield a fragment with a terminal, reducible

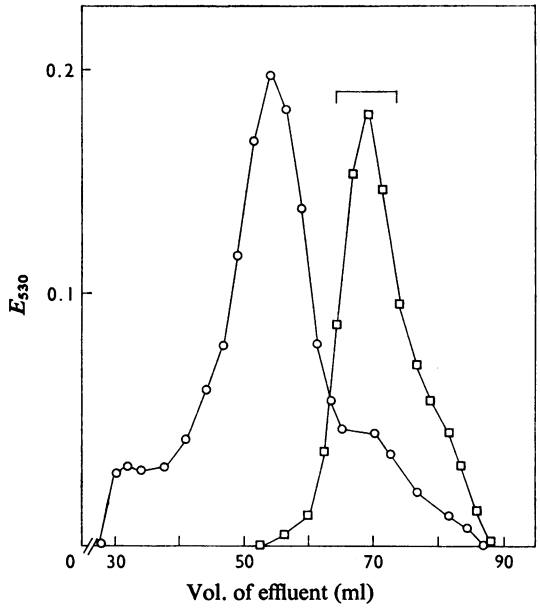


Fig. 5. *Gel chromatography on Sepharose 4B of dermatan sulphate, before* (○) *and after* (□) *digestion with papain*

The analysed material was the high-molecular-weight portion of the 0.5M-light fraction (see Fig. 3*b*). The fractions of the papain-digested material indicated by the horizontal bracket were pooled and subjected to further analysis. For further details see the text.

sugar residue. The presence of such residues was demonstrated by reduction of liver-capsule heparin with NaB^3H_4. The amount of ^3H incorporated was related to the molecular weight of the heparin, yielding a specific radioactivity of 22mCi/mmol of polysaccharide, or 1.3 reducible groups per polysaccharide chain (based on a specific radioactivity for the NaB^3H_4 used of 68mCi/mmol, calculated from the $^3H/^{14}C$ ratio of the sorbitol internal standard). This latter value should be regarded as approximate, as several of the parameters involved in the calculations may have been subject to experimental error.

The ^3H-labelled monosaccharide residue was identified after degradation of the reduced polysaccharide by a combination of acid hydrolysis and deaminative cleavage with HNO_2 (see the Experimental section). The products (recovery of radioactivity from labelled heparin, 80%) were analysed by paper electrophoresis at pH5.3 (Fig. 8*a*), showing a major component migrating like D-glucuronic acid, L-iduronic acid or L-gulonic acid (which all show the same migration rate under these conditions). On electrophoresis at the lower pH3.9 (Fig. 8*b*) the charged, labelled component was retarded in relation to the uronic acids, and migrated similarly to the L-gulonic acid standard. These results suggest that the labelled sugar residue was an aldonic acid; owing to lack of material it was not possible to identify this component further.

An aldonic acid (L-gulonic acid or L-idonic acid) would be the expected reduction product of a terminal uronic acid (D-glucuronic acid or L-iduronic acid respectively) residue. Results of model experiments to be reported elsewhere Ögren & Lindahl, 1975) demonstrated that a glucuronic acid residue in the reducible terminal position of a polysaccharide chain was in fact recovered as [^3H] gulonic acid by the procedure employed. Glucosamine residues in a similar position were largely converted into an uncharged, labelled deamination product; a smaller fraction appeared as a component

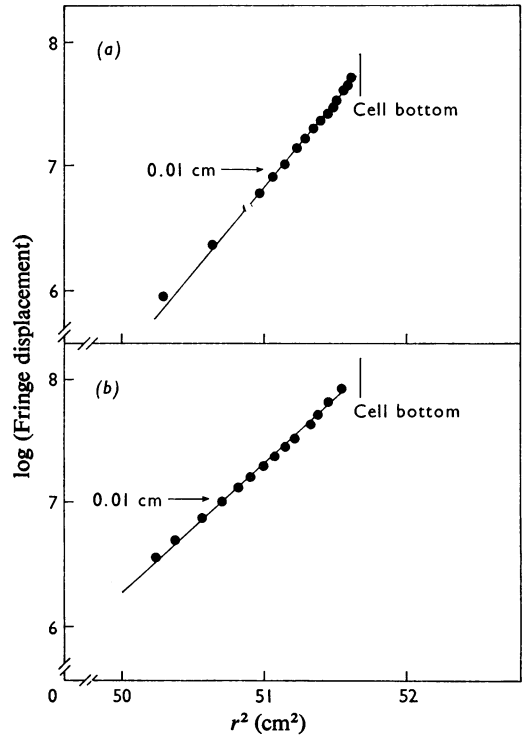


Fig. 6. *Molecular-weight determination of heparin and papain-digested dermatan sulphate*

The molecular weights were determined by sedimentation-equilibrium ultracentrifugation of (*a*) heparin (pooled fractions in Fig. 4*a*) at 36000rev./min and (*b*) papain-digested dermatan sulphate (pooled fractions in Fig. 5) at 22000rev./min. The logarithm of fringe displacement (measured in cm$\times 10^{-4}$) is plotted against the square of the radius (r) measured in cm.

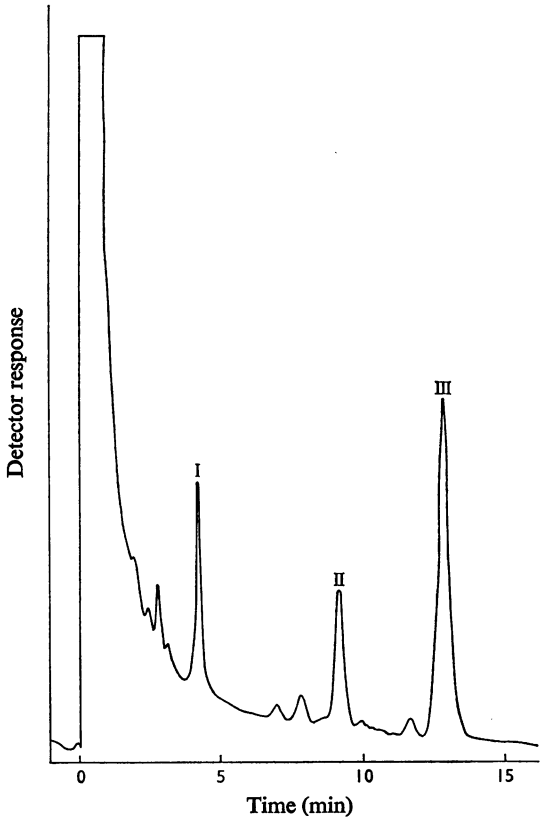


Fig. 7. *Gas–liquid chromatography of neutral sugar derivatives from heparin*

I, Xylose; II, galactose; III, inositol (internal standard).

migrating like the charged disaccharide, glucuronosyl-anhydromannitol. As seen from Fig. 8(*a*) only minute amounts of radioactivity appeared in the disaccharide position; further, most of the uncharged labelled material represented aldonolactone.

Taken together, the results obtained strongly indicate that most of the heparin molecules in bovine liver capsule have a terminal, reducible uronic acid residue. It therefore seems reasonable to conclude that these polysaccharide fragments have been liberated from a larger molecule by an endo-glycuronidase.

Discussion

The results of the present study show that heparin in bovine liver capsule occurs largely (or exclusively) as single polysaccharide chains. Only about 15% of these chains contain the polysaccharide–protein linkage region, the majority of the molecules instead having terminal, reducible uronic acid residues. These results confirm and extend our previous findings (Lindahl, 1970), and are clearly at variance with the reports of Serafini-Fracassini *et al.* (1969, 1973), who claimed that most of the heparin in bovine liver capsule occurs as a macromolecular complex, consisting of two polysaccharide chains joined by a polypeptide. In consequence with these claims, it was stated that all heparin chains contained covalently bound xylose and galactose, and further, that the relative lack of xylose reported in our previous study (Lindahl, 1970) must reflect degradation occurring during isolation. Such degradation appears highly improbable, in view of the following facts. (*a*) The isolation procedure employed in the present investigation has been extensively, and successfully, applied to the isolation of intact proteoglycans from several types of tissues (Antonopoulos *et al.*, 1974); although this procedure differs considerably from that used in our previous study (Lindahl, 1970), similar low xylose, galactose and serine contents were recorded for the isolated heparin products, irrespective of the preparation procedure used. (*b*) Dermatan sulphate, isolated along with the heparin from the same tissue, retained the macromolecular properties of a proteoglycan during the isolation procedure. (*c*) Isolated chains of

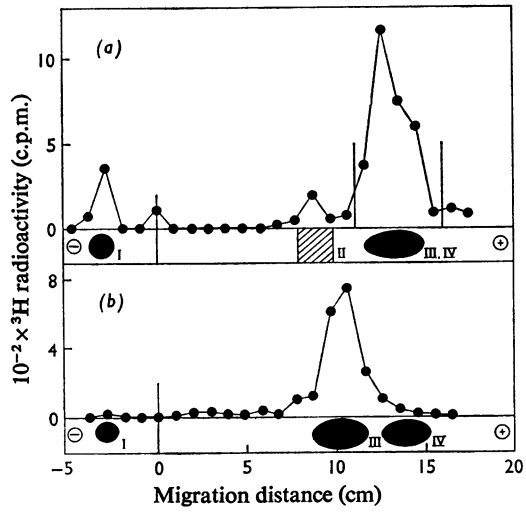


Fig. 8. *Identification of reducible end group in heparin*

Paper electrophoresis at (*a*) pH 5.3 and (*b*) pH 3.9 of labelled components derived from heparin (pooled fractions in Fig. 4*a*), reduced with NaB^3H_4. The material subjected to electrophoresis at pH 3.9 was obtained by elution of the charged component appearing between the vertical lines in (*a*). I, Lactones of L-gulonic acid and D-glucuronic acid; II, [^{14}C]glucuronosyl-anhydromannitol; III, L-gulonic acid; IV, D-glucuronic acid.

dermatan sulphate contained one residue of xylose per molecule, in contradiction of the possibility that polysaccharide-bound xylose would be selectively lost as a result of the isolation conditions.

Degradation does not occur during isolation and therefore the most reasonable explanation for the lack of polysaccharide–protein linkage region in the liver-capsule heparin would seem to be degradation of the polysaccharide *in vivo*. The resulting fragments were shown to contain uronic acid residues in reducing, terminal position: hence, the enzyme responsible for the degradation should be an endo-glycuronidase. This conclusion is of interest in relation to previous studies on the metabolism of heparin in rodents. Heparin isolated from rat skin occurs as a complex, branched macromolecule, composed of several glycosaminoglycan chains joined by a polysaccharide core (Horner, 1971). Newly synthesized heparin from neoplastic mouse mast cells displays similar macromolecular properties (Ögren & Lindahl, 1971). These macromolecular heparins may be depolymerized by endoglycosidases from various rodent (Ögren & Lindahl, 1971; Horner, 1972; S. Ögren & U. Lindahl, unpublished work) and human (S. Ögren, unpublished work) tissues, yielding fragments similar in size to commercial heparin. In the mouse mastocytoma, biosynthesis and degradation of the macromolecular heparin occur in rapid succession (Ögren & Lindahl, 1971; S. Ögren & U. Lindahl, unpublished work); it has been suggested by Horner (1972) that the degradation process may be required to convert the heparin into its physiologically active form. The enzyme responsible for the degradation of macromolecular heparin in mouse mastocytoma has been isolated and tentatively identified as an endoglucuronidase (Ögren & Lindahl, 1975). These findings, in conjunction with the results of the present study, suggest that formation and degradation of macromolecular heparin may occur also in mammals other than rodents (see also Horner, 1970).

This work was supported by grants from the Swedish Medical Research Council (13X-2309; 13P-3431), the Swedish Cancer Society (53), Gustaf V:s 80:årsfond and the Medical Faculty, University of Uppsala. We are indebted to Dr. H. Pertoft, who carried out the ultracentrifugation analyses. The excellent technical assistance of Miss I. Pettersson is gratefully acknowledged.

References

Antonopoulos, C. A., Axelsson, I., Heinegård, D. & Gardell, S. (1974) *Biochim. Biophys. Acta* **338**, 108–119
Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
Blix, G. (1941) *J. Biol. Chem.* **137**, 495–501
Chervenka, C. H. (1970) *Anal. Biochem.* **34**, 24–29
Edebo, L. (1960) *J. Biochem. Microbiol. Technol. Engng.* **2**, 453–479
Höök, M., Lindahl, U. & Iverius, P. H. (1974) *Biochem. J.* **137**, 33–43
Horner, A. A. (1970) *Proc. Can. Fed. Biol. Soc.* **13**, 109
Horner, A. A. (1971) *J. Biol. Chem.* **246**, 231–239
Horner, A. A. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 3469–3473
Jansson, L. & Lindahl, U. (1970) *Biochem. J.* **117**, 699–702
Kimmel, J. R. & Smith, E. L. (1954) *J. Biol. Chem.* **207**, 515–531
Koeltzow, D. E., Epley, J. D. & Conrad, H. E. (1968) *Biochemistry* **7**, 2920–2928
Kraemer, P. M. & Smith, D. A. (1974) *Biochem. Biophys. Res. Commun.* **56**, 423–430
Lasker, S. E. & Stivala, S. S. (1966) *Arch. Biochem. Biophys.* **115**, 360–372
Lindahl, U. (1970) *Biochem. J.* **116**, 27–34
Lindahl, U. & Rodén, L. (1972) in *Glycoproteins* (Gottschalk, A., ed.), vol. A, pp. 491–517, Elsevier Publishing Co., Amsterdam
Lindahl, U., Cifonelli, J. A., Lindahl, B. & Rodén, L. (1965) *J. Biol. Chem.* **240**, 2817–2820
Lindahl, U., Bäckström, G., Jansson, L. & Hallén, A. (1973) *J. Biol. Chem.* **248**, 7234–7241
Lloyd, A. G., Bloom, G. D., Balasz, E. A. & Haegermark, Ö. (1967*a*) *Biochem. J.* **103**, 75P–76P
Lloyd, A. G., Bloom, G. D. & Balasz, E. A. (1967*b*) *Biochem. J.* **103**, 76P–77P
Lohmander, S. (1972) *Biochim. Biophys. Acta* **264**, 411–417
Luscombe, M. & Phelps, C. F. (1967) *Biochem. J.* **103**, 103–109
Ögren, S. & Lindahl, U. (1971) *Biochem. J.* **125**, 1119–1129
Ögren, S. & Lindahl, U. (1975) *J. Biol. Chem.* in the press
Sawardeker, J. S., Sloneker, J. H. & Jeanes, A. (1965) *Anal. Chem.* **37**, 1602–1604
Serafini-Fracassini, A., Durward, J. J. & Floreani, L. (1969) *Biochem. J.* **112**, 167–172
Serafini-Fracassini, A., Branford-White, C. J. & Hunter, J. C. (1973) *FEBS Lett.* **32**, 116–118
Smith, I. (1960) *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 252, Interscience Publishers, New York
Wessler, E. (1968) *Anal. Biochem.* **26**, 439–444
Wessler, E. (1971) *Anal. Biochem.* **41**, 67–69