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Evidence for the Existence of a Multichain Proteoglycan of Heparan Sulphate

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1. Glycosaminoglycans were extracted with 2M-potassium chloride from bovine aorta and purified by precipitation with cetylpyridinium chloride from 0.5M-potassium chloride. The yield amounted to 24% of the total glycosaminoglycan content of the tissue. 2. After removal of chondroitin sulphate by digestion with testicular hyaluronidase, the residual glycosaminoglycan material (11% of the extracted polysaccharide) was fractionated by gel chromatography on Sephadex G-200. Two peaks (I and II) were obtained, the more retarded of which (II) corresponded to single polysaccharide chains. 3. The macromolecular properties of fraction I were investigated by repeated gel chromatography, after treatment of the fraction with alkali or digestion with papain. In both cases the elution position of fraction I was shifted towards that of the single polysaccharide chains. 4. Analysis of fraction I showed approximately equal amounts of heparan sulphate and dermatan sulphate. It is concluded that these glycosaminoglycans both occur in the aortic wall as multichain proteoglycans.

The glycosaminoglycans of connective tissues are generally covalently bound to protein, forming proteoglycans consisting of several polysaccharide chains linked to a polypeptide backbone. The glycosaminoglycan constituents of such macromolecules (termed 'multichain proteoglycans' below) have been identified as chondroitin 4-sulphate, chondroitin 6-sulphate, keratan sulphate (Barrett, 1968) and dermatan sulphate (Toole & Lowther, 1968).

All the uronic acid-containing proteoglycans mentioned above contain the same polysaccharide–protein linkage region, involving the trisaccharide D-galactosyl-D-galactosyl-D-xylose in a glycosidic linkage with the hydroxyl group of serine (Rodén, 1968; Helting & Rodén, 1968; Fransson, 1968). The latter linkage is characteristically susceptible to alkali, which effects the liberation of single polysaccharide chains by a β-elimination mechanism (Anderson, Hoffman & Meyer, 1965). Thus, a multichain proteoglycan may conveniently be detected by the decrease in size occurring during treatment with alkali. Conversion into a single-chain state may also be effected by degrading the protein component of the molecule with proteolytic enzymes. By the use of these techniques the present investigation demonstrates that the group of glycosaminoglycans occurring in the native state as multichain proteoglycans also includes heparan sulphate.

EXPERIMENTAL PROCEDURE AND RESULTS

Materials. The ascending portions of aortae from freshly slaughtered cows were dissected free of adventitia, homogenized by repeated passage through a bacteria press at −20°C (Edebo, 1960) and dried with acetone–ether (Blix, 1941).

The glycosaminoglycans used as reference standards were as described by Lindahl (1970).

Testicular hyaluronidase (EC 3.2.1.35; 14000i.u./mg) was purchased from AB Leo, Hälsingborg, Sweden. Crystalline papain (EC 3.4.4.10) was prepared 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. 40mg of protein/ml. Ribonuclease (EC 2.7.7.16; type I-A) and deoxyribonuclease (EC 3.1.4.5) were purchased from Sigma Chemical Co.

Analytical methods. Uronic acid was determined by the method of Bitter & Muir (1962). Analyses of total hexosamine were performed by a modification (Gardell, 1953) of the Elson–Morgan reaction, after hydrolysis of polysaccharide samples in 4M-HCl for 14h. Glucosamine/galactosamine ratios were determined with a Bio-Cal model B-200 amino acid analyzer.

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, 1969). 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. Samples (1 ml) of polysaccharides were mixed with equal volumes of 4 M-KCl and applied to a column (0.9 cm × 100 cm) of Sephadex G-200. Elution was carried out with 2 M-KCl, at a rate of approx. 4 ml/h. Effluent fractions were analysed for uronic acid by the carbazole reaction.

Extraction and fractionation of polysaccharides. Portions (50 g) of homogenized and dried aorta were suspended in 900 ml of ice-cold 2 M-potassium chloride and extracted with continuous stirring for 30 min. After removal of insoluble material by centrifugation, the extract was mixed with an equal volume of 1% (w/v) CPC* in water and diluted to a final concentration of 0.5 M with respect to potassium chloride. The precipitated cetylpyridinium-polysaccharide was collected on a Celite pad, extracted in 2 M-sodium chloride–10% (v/v) ethanol and finally converted into the sodium salt of the polysaccharide (fraction A), as described by Lindahl (1970). The uronic acid content of fraction A corresponded to 0.06% of the dry weight of the aorta and to 24% of the total glycosaminoglycan, isolated as described above after digestion of the tissue with papain (Lindahl, 1970). Appreciable amounts of nucleic acids were present, as indicated by the high viscosity of fraction A in solution and by the extinction peak at 260 nm. The yield of polysaccharide was not significantly increased by prolonged or repeated extraction procedures.

The polysaccharide constituent of fraction A was analysed by electrophoresis in barium acetate (Fig. 1*a*). After staining with Alcian Blue, three spots were detected, corresponding to chondroitin sulphate, dermatan sulphate and heparan sulphate (or heparin). Chondroitin sulphates and nucleic acids were eliminated by digestion of fraction A (25 mg of uronic acid) at 37°C for 12 h with a mixture of 5 mg of testicular hyaluronidase, 3 mg of ribonuclease and 3 mg of deoxyribonuclease in 7 ml of 0.05 M-tris–HCl–0.15 M-sodium chloride–0.02 M-magnesium chloride, pH 7.0. The hyaluronidase-resistant polysaccharides (fraction A-hyase), accounting for 11% of the uronic acid content of fraction A, were reisolated by precipitation with CPC from 0.5 M-potassium chloride as described above. Electrophoresis in barium acetate of fraction A-hyase (Fig. 1*a*) showed spots of dermatan sulphate and heparan sulphate (or heparin) whereas chondroitin sulphate was not detected.

Macromolecular properties of glycosaminoglycans. The macromolecular properties of fraction A-hyase were investigated by chromatography on Sephadex G-200, as shown in Fig. 2. Two peaks of uronic acid-containing material were observed. The retarded elution position of fraction A-hyase-II suggested the presence of single polysaccharide chains or polysaccharide–peptides; the average molecular weight would be approximately 8000–10000, as indicated by a comparison with gel chromatograms of calibrated fractions of chondroitin 4-sulphate (Wasteson, 1969). The behaviour on gel chromatography was not affected by treatment of the material with alkali or with papain, thus further indicating the absence in fraction A-hyase-II of multichain proteoglycans. It should be observed, though, that some *O*-glycosyl serine linkages are split by alkali only with difficulty (Neuberger, Gottschalk & Marshall, 1966; Derevitskaya, Vafina & Kochetkov, 1967). In contrast to fraction A-hyase-II, the material excluded from Sephadex G-200 [fraction A-hyase-I; minimum mol.wt. 70000 (Å. Wasteson, personal communication)] was found to be largely degraded (fraction A-hyase-I, OH^- in Fig. 2*b*) after treatment with alkali (0.5 M-sodium hydroxide at

* Abbreviation: CPC, cetylpyridinium chloride.

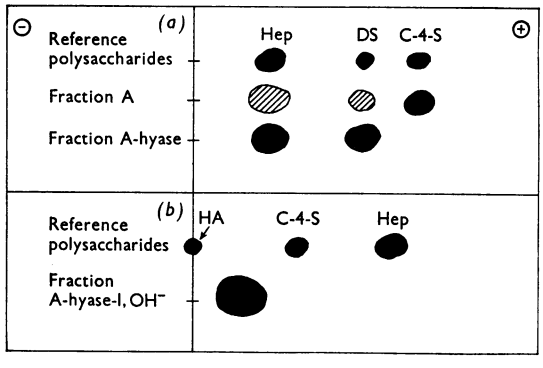

Fig. 1. Electrophoresis on cellulose acetate of polysaccharide fractions in (*a*) 0.1 M-barium acetate or (*b*) 0.1 M-HCl. C-4-S, chondroitin 4-sulphate; HA, hyaluronic acid; DS, dermatan sulphate; Hep, heparin. The electrophoresis patterns of fractions A-hyase-I, A-hyase-II and A-hyase-I,OH^- were similar to that of fraction A-hyase. All fractions were treated with alkali before electrophoresis, as indicated in the text. Electrophoresis in barium acetate of polysaccharides not subjected to alkaline conditions resulted in appreciable streaking of fraction A-hyase and, in particular, of fraction A-hyase-I. Fraction A-hyase-II showed similar electrophoresis patterns before and after treatment with alkali. Electrophoresis in 0.1 M-HCl was only performed with the alkali-treated material.

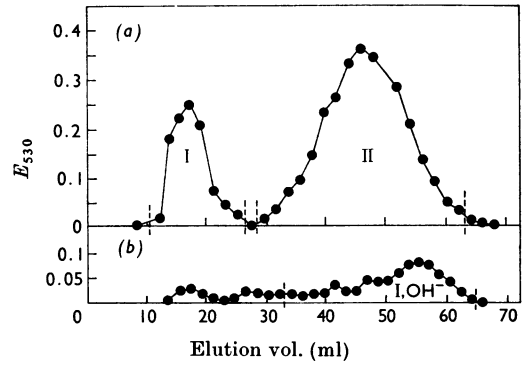

Fig. 2. Gel chromatography on Sephadex G-200 of (*a*) fraction A-hyase, (*b*) fraction A-hyase-I after treatment with alkali. Effluent fractions were analysed for uronic acid by the carbazole reaction and combined as indicated by the dashed lines.

4°C for 20h). A similar degradation, also demonstrated by gel chromatography, was achieved by digestion with papain (Lindahl, 1970), thus corroborating the presence in fraction A-hyase-I of a multichain proteoglycan.

Electrophoresis in barium acetate of the polysaccharide constituents of fractions A-hyase-I and A-hyase-II in both cases showed spots corresponding to dermatan sulphate and heparan sulphate (or heparin), respectively. The same electrophoresis pattern was produced by the single polysaccharide chains liberated from fraction A-hyase-I by alkali (fraction A-hyase-I,OH^-). The presence of heparan sulphate in fraction A-hyase-I,OH^- was further substantiated by hexosamine analysis, which showed glucosamine to constitute 50% of the total hexosamine. The uronic acid/hexosamine molar ratio was 0.84, the somewhat low uronic acid value probably reflecting the presence of iduronic acid in dermatan sulphate (Bitter & Muir, 1962). Electrophoresis in 0.1M-hydrochloric acid showed that the polysaccharide components of fraction A-hyase-I,OH^- migrated somewhat more slowly than chondroitin sulphate, which was in turn considerably retarded in relation to a sample of commercial heparin (Fig. 1*b*). From the latter experiment it is concluded that the glucosamine-containing, hyaluronidase-resistant glycosaminoglycan in fraction A-hyase-I,OH^- is heparan sulphate and not heparin. Judging from the uronic acid/hexosamine ratio, the amount of keratan sulphate present, if any, must be negligible.

DISCUSSION

The macromolecular properties of the native α-linked glycosaminoglycans, heparin and heparan sulphate, have been unclear. Both polysaccharides contain residual peptides when isolated after proteolytic digestion of tissues (Lindahl, Cifonelli, Lindahl & Rodén, 1965; Jacobs & Muir, 1963); the peptides are linked to the polysaccharide chains proper by the specific neutral trisaccharide mentioned above (Lindahl & Rodén, 1965; Lindahl, 1966*a*,*b*, 1967; Knecht, Cifonelli & Dorfman, 1967). Although these findings are compatible with the occurrence of multichain proteoglycans of heparin and heparan sulphate, the existence of such macromolecules has not hitherto been established. Recent attempts at isolating heparin proteoglycans from bovine liver capsule (Lindahl, 1970) and mouse mastocytoma (U. Lindahl & S. Ögren, unpublished work) yielded single polysaccharide chains and polysaccharide–peptides but no multichain compounds. Pulse labelling *in vivo* of mastocytoma heparin with [^{35}S]sulphate in fact showed that the newly-synthesized polysaccharide chain is rapidly degraded to products of lower molecular weight; analysis of the degraded fragments implicated the involvement of a polysaccharidase with endo-enzyme properties.

The results of the present investigation suggest that the macromolecular properties of native heparan sulphate differ from those of heparin. In contrast to the latter polysaccharide heparan sulphate occurs as a macromolecule which behaves on treatment with alkali or proteolytic enzymes as does a multichain proteoglycan. The occurrence of single polysaccharide chains or polysaccharide–peptides of heparan sulphate and dermatan sulphate in addition to the corresponding multichain proteoglycans may be due to the presence, in the tissue, of enzymes with protease or endo-polysaccharidase activities. The biosynthesis of single polysaccharide–peptides cannot be excluded, but seems a less likely alternative. It should be noted that the extraction procedure used preferentially yields low-molecular-weight components; therefore, the relative amounts of fractions A-hyase-I and A-hyase-II do not reflect the multichain proteoglycan/single polysaccharide chains ratio in the tissue. An attempt was made to increase the yield of proteoglycan by modifying the extraction procedure. Extraction of the homogenized aorta with 6M-urea at 60°C for 20h brought about a threefold increase in the yield of carbazole-reacting material, as compared with the extraction with 2M-potassium chloride. In contrast to the salt-extractable glycosaminoglycans, the polysaccharide extracted with urea was largely excluded from Sephadex G-200. Part of the excluded material, after treatment with alkali, migrated like heparan sulphate on electrophoresis in barium acetate.

The complete separation of the dermatan sulphate and heparan sulphate proteoglycans is a prerequisite for the further characterization of the latter macromolecule.

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