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Isolation and characterization of dermatan sulphate proteoglycan from human uterine cervix

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Proteoglycans were extracted from human uterine cervix with 4 M-guanidinium chloride in the presence of proteinase inhibitors. They were purified by density-gradient centrifugation in 4 M-guanidinium chloride/CsCl (starting density 1.32 g/ml) followed by DEAE-cellulose and Sepharose chromatography. Only one polydisperse proteoglycan was found. $s^0_{20,w}$ was 2.1 S and the weight-average molecular weight was 73 000 (sedimentation-equilibrium centrifugation) to 110 500 (light-scattering). The core protein was monodisperse, with an apparent molecular weight of 47 000. The proteoglycan contained about 30% protein and probably two or three glycosaminoglycan side chains per molecule. High contents of aspartate, glutamate and leucine were found. The glycan moiety of the proteoglycan was exclusively dermatan sulphate, with a co-polymeric structure with approximately equal quantities of iduronic acid- and glucuronic acid-containing disaccharides.

More than 90% of the human uterine cervix is made up by fibrous connective tissue (Rorie & Newton, 1967). Collagen of types I and III (Kleissl *et al.*, 1978), together with hyaluronic acid, heparan sulphate and dermatan sulphate, are typical fibrous connective-tissue components found in the tissue (for review see Ellwood & Anderson, 1981). The uterine cervix changes its properties during pregnancy. From being hard 'as cartilage' in the non-pregnant state, the organ softens during pregnancy to become easily distensible at the time for delivery. This change (ripening) can also be induced by prostaglandin E_2 applied in the cervical canal (Forman *et al.*, 1982). It has been verified that the ripening is caused primarily by changes in the connective tissue, and that it is independent of muscular contractions (Forman *et al.*, 1982). The uterine cervix can therefore be a useful model for study of the physiology of connective tissue. In order to achieve this it is important to know the composition of the tissue and the structure of the various components. As little is known about the proteoglycans of the cervix, the present work was started in order to characterize these components.

Experimental

Materials

Uterine cervical tissue from nine post-climacteric patients with a mean age of 63 years (range 53–71 years) was used. Four of them were operated with supravaginal hysterectomy for benign myomas, and five with amputation of cervix in connection with vaginal repair of prolapsis uteri. The cervical epithelium was cut away with a scalpel, and special care was taken to remove mucus-filled crypts. The stroma was immersed immediately into liquid N_2 and was then stored at −60°C.

Sepharose and Sephadex gels, reference proteins (high-molecular-weight range and low-molecular-weight range) and Blue Dextran were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). DEAE-cellulose (DE-52) ion-exchanger was from Whatman (Maidstone, Kent, U.K.). Papain (EC 3.4.22.2) (twice-crystallized, 16–40 Bz-Arg-OEt units/mg) and guanidinium chloride (practical grade) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chondroitinase ABC (EC 4.2.2.4) and chondroitinase AC (EC 4.2.2.5) were obtained from

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Miles Laboratories (Elkhart, IN, U.S.A.). Dialysis tubing (molecular-weight cut-off 6000–8000) was from Spectrum Medical (Los Angeles, CA, U.S.A.). Guanidinium chloride was treated with activated charcoal and filtered to obtain an A_{280} below 0.13 for an 8 M solution. Solutions of CsCl and $CsSO_4$ were treated in the same way to remove a slight haze from the solution. Stock solutions of urea (7 M) were passed through a mixed anion/cation-exchange resin to remove traces of cyanate immediately before use.

Analytical methods

Hexosamines were determined by a modification (Antonopoulos *et al.*, 1964) of the Elson–Morgan procedure. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and hexuronic acid was determined by the orcinol method (Brown, 1946) or the carbazole/borate method (Bitter & Muir, 1962). An automated version of the latter method was also employed (Heinegård, 1973). Glucosamine, galactosamine and amino acids were determined with a Durrum amino acid analyser. Hydrolyses were performed (under argon) in 4 M-HCl at 100°C for 10h (hexosamine) or in 6 M-HCl at 110°C for 24h (amino acids). The dry weight used for calculation of the water content was obtained by keeping samples *in vacuo* over P_2O_5 for 60h.

Neutral sugars were determined by g.l.c. of their alditol acetates after hydrolysis in 2 M-trifluoroacetic acid at 100°C for 5h (Axelsson & Heinegård, 1975).

Analytical ultracentrifugation and light-scattering

Determinations of the weight-average molecular weight by meniscus-depletion sedimentation-equilibrium centrifugation and of the sedimentation coefficient were performed as described by Cöster *et al.* (1981), and the isopycnic density-gradient ultracentrifugations as described by Sheehan *et al.* (1981).

Proteoglycan was dissolved in 4 M-guanidinium chloride/1 mM-sodium phosphate/1 mM-EDTA, pH 6.5, for light-scattering experiments. Details of clarification of solutions, instrument used for measurement of scattered light, as well as methods of calculations, have been described by Fransson *et al.* (1979). The apparent refractive-index increment at constant chemical potential was determined at 436nm and 20°C with a Shimadzu model DR-4 differential refractometer on a solution containing approx. 4 mg of proteoglycan/ml.

Degradation methods and separation methods

Digestions with papain, chondroitinase ABC and chondroitinase AC were performed essentially as described by Heinegård *et al.* (1981).

Periodate oxidation/alkaline elimination (selective cleavage of L-iduronic acid residues) at pH 3.0 and 4°C was used to degrade liberated dermatan sulphate side chains as described previously (Fransson & Cöster, 1979).

Separation of the oligosaccharides and the glycosaminoglycans was accomplished by chromatography of a papain digest of the proteoglycan on a DEAE-cellulose column. Oligosaccharides were eluted with 20 mM-HCl and freeze-dried. The glycosaminoglycans were eluted with 2 M-NaCl, dialysed against water and freeze-dried.

Polyacrylamide-gel electrophoresis conducted on 8% (w/v) gels in the presence of 0.1% sodium dodecyl sulphate by the procedure of Neville (1971) was performed as described by Carlstedt *et al.* (1981). The gels were stained with 0.25% Kenacid or periodate/Schiff reagent (Zacharius *et al.*, 1969).

Extraction and isolation of proteoglycan

Cervical tissue (37.9g) was powdered, extracted with 4 M-guanidinium chloride containing proteinase inhibitors (10 mM-6-aminohexanoic acid, 10 mM-EDTA, 5 mM-benzamidinium chloride and 5 mM-*N*-ethylmaleimide), and subjected to CsCl-density-gradient centrifugation, ion-exchange chromatography and chromatography on Sepharose CL-4B as described by Cöster & Fransson (1981) and in the respective Figure legends.

Results and discussion

Extraction and isolation of cervical proteoglycan

The proteoglycan was extracted from the powdered tissue with 4 M-guanidinium chloride in the presence of proteinase inhibitors. *N*-Ethylmaleimide also prevented disulphide exchange. Density-gradient centrifugation of the extract distributed the sulphated glycosaminoglycan-containing material from the bottom fraction up to a density of 1.30 mg/ml (Fig. 1). Most of the protein (estimated as the absorbance at 280nm) was obtained at lower densities. The bottom six fractions were pooled, dialysed and subjected to ion-exchange chromatography (Fig. 2). Mucins were eluted at low acetate concentration (Uldbjerg *et al.*, 1982*b*) and thus effectively separated from the proteoglycans, which were obtained at higher ionic strength. The proteoglycans were pooled as shown in Fig. 2 and subjected to gel chromatography on Sepharose CL-4B (Fig. 3), which afforded the separation of one polydisperse proteoglycan from nucleic acid. After freeze-drying, 25.6 mg of proteoglycan (sodium salt) was obtained. The overall yield after these steps was 60% of the amount of dermatan sulphate that can be isolated after ion-exchange chromatography of a papain digest of identical tissue material (Uldbjerg *et al.*, 1982*b*).

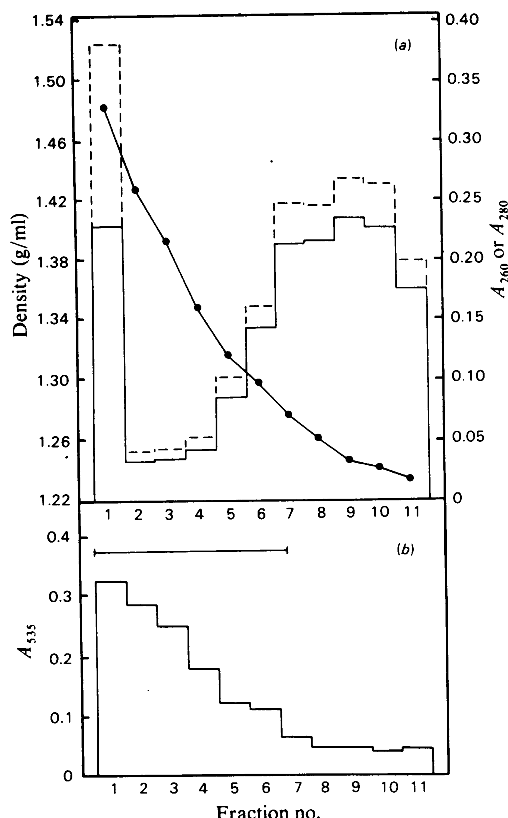

Fig. 1. *Guanidinium chloride-(4* M*)/CsCl-density-gradient centrifugation (starting density 1.32 g/ml) of uterine cervix extract*

The tubes were emptied (2 ml fractions) from the bottom. (*a*) ——, A_{280}; ----, A_{260}; ●—●, density. (*b*) Sulphated glycosaminoglycans quantified as hexosamine (A_{535}) after an isolation proceduce including papain digestion and ion-exchange chromatography (Uldbjerg *et al.*, 1982*a*). Fractions were pooled as indicated by the horizontal bar (fractions 1–6).

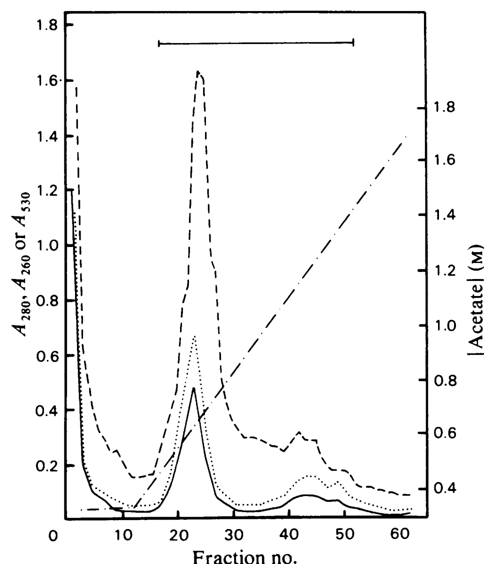

Fig. 2. *Ion-exchange chromatography of the material with densities greater than 1.30 g/ml on density-gradient centrifugation*

The material was dialysed against 6 M-urea/0.4 M-sodium acetate buffer, pH 5.8, and passed through a DEAE-cellulose column (1 cm × 24 cm) equilibrated with the same buffer. The elution was performed with a linear gradient: mixing vessel, 200 ml of 6 M-urea/0.4 M-sodium acetate buffer, pH 5.8; reservoir, 200 ml of 6 M-urea/1.5 M-sodium acetate buffer, pH 5.8. The fraction volume was 5 ml. ——, A_{280}, ·····, A_{260}; ----, uronic acid (A_{530}, carbazole method); ·–·, concentration of acetate (conductivity determination). Fractions were pooled as indicated by the horizontal bar (fractions 17–52).

Physical characterization of the proteoglycan

Analytical isopycnic ultracentrifugation in a pure CsCl gradient showed only one sharp u.v.-absorbing component banding at a density of 1.65 mg/ml (Fig. 4). The proteoglycan has a low absorption coefficient at 280 nm (about 0.5 mg^{-1}). DNA, which bands at 1.7 mg/ml and has a considerably higher absorption coefficient, can therefore be excluded as a major impurity. The presence of small amounts of protein at the meniscus cannot be excluded by this method. Isopycnic ultracentrifugation in a pure Cs_2SO_4 gradient showed no material at the meniscus. The material banded asymmetrically around $\rho = 1.44$ g/ml. The observed heterogeneity is probably due to polydispersity both in the iduronic acid content of the dermatan sulphate and in the polysaccharide/protein ratios (Sheehan *et al.*, 1981), but not to the presence of DNA.

Molecular-weight determinations were performed in 4 M-guanidinium chloride (Cöster *et al.*, 1981). Light-scattering data were treated in accordance with the Zimm (1948) procedure. The value for dn/dc was 0.146, which gave a molecular weight of 110 500 and a radius of gyration of 43 nm.

The partial specific volume was determined to be 0.58 ml/g. In sedimentation-equilibrium experiments a linear relationship between ln c and r^2 was obtained for the three concentrations 0.206 mg/ml, 0.619 mg/ml and 0.825 mg/ml. Extrapolation of the apparent molecular weights to zero concentration gave a value of 72 000. In the sedimentation-velocity experiments the material sedimented as a single component at five different concentrations between

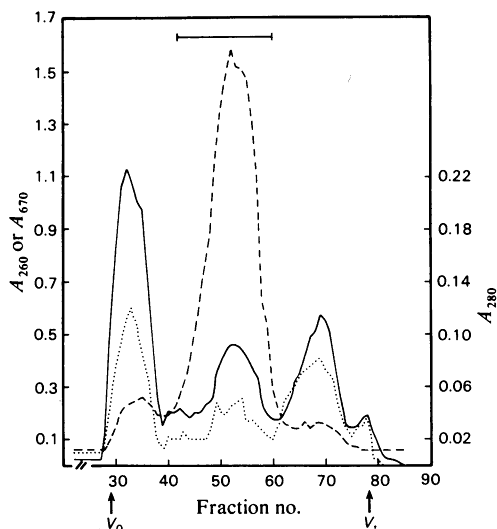

Fig. 3. *Sepharose CL-4B gel chromatography of the material pooled after DEAE-cellulose chromatography*
The material was concentrated in an Amicon cell with a PM 10 filter and applied on a Sepharose CL-4B column (150cm × 1.6cm) eluted with 4M-guanidinium chloride/1mM-sodium phosphate/1mM-EDTA, pH6.5. The fraction volume was 3.9ml. ——, A_{280}; ·····, A_{260}; ----, uronic acid (A_{670}, orcinol method). Fractions were pooled as indicated by the horizontal bar (fractions 42–60). V_0, Void volume; V_t, total volume.

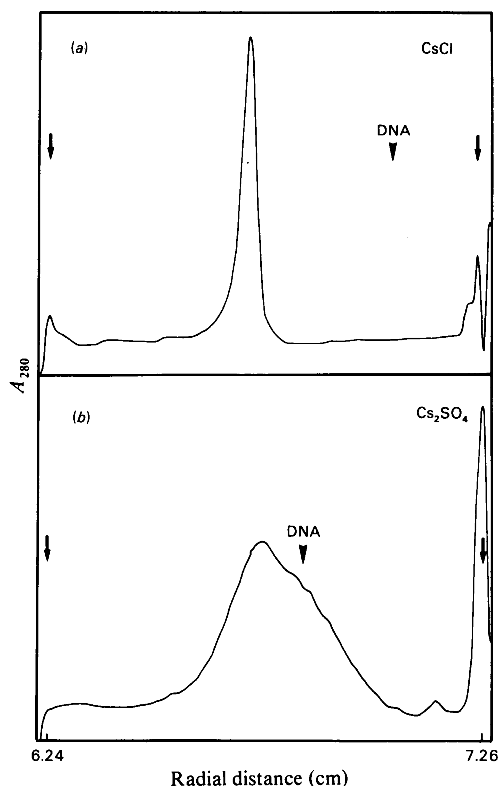

Fig. 4. *Analytical density-gradient centrifugations of purified proteoglycan (a) in CsCl and (b) in Cs_2SO_4*
(*a*) Centrifugation was performed in a CsCl gradient at a starting density of 1.655g/ml and a rotor speed of 57000rev./min (Centriscan 75) at 25°C. (*b*) Centrifugation was performed in a Cs_2SO_4 gradient at a starting density of 1.45g/ml and a rotor speed of 50000 rev./min (Centriscan 75) at 25°C. The meniscus and the bottom of the tubes are marked with ↓ and the position where DNA would band is marked with ▼.

1.31mg/ml and 3.93mg/ml. By using these data $s^0_{20,w}$ was calculated to be 2.1S.

The half-width of the peak obtained under isopycnic ultracentrifugation gives a rough estimation of the molecular weight (Sheehan *et al.*, 1981). By this method weight-average molecular weights of 4.6×10^6 and 106000 (by use of the left half of the peak) were found in CsCl and Cs_2SO_4 respectively (Fig. 4). Large aggregates in CsCl may explain this difference (Sheehan *et al.*, 1981).

It is known that dermatan sulphate proteoglycans form aggregates due to self-association independent of hyaluronic acid (Cöster *et al.*, 1981). Attempts to prevent the formation of such aggregates by working in 4M-guanidinium chloride may not have been entirely successful. The shape of the Zimm plot at low angles (30–60°) suggests that the presence of small amounts of aggregates may be giving a slightly high weight-average molecular weight in the light-scattering experiments. In the sedimentation-equilibrium experiments in the analytical ultracentrifuge, such residual aggregates will tend to be centrifuged to the bottom of the cell in the experiment of the Yphantis (1964) type, thus leaving the molecular-weight determination undisturbed. This probably explains the higher molecular weight obtained in the light-scattering experiments.

Chemical characterization of the proteoglycan

The overall chemical composition is given in Table 1. The protein content was between 42% (determined by the method of Lowry *et al.*, 1951) and 29% (determined on the amino acid analyser) by weight. The contents of serine and threonine were low, whereas those of leucine, asparate and glutamate were high. Contamination by collagen is excluded, as no hydroxyproline was detected. Galactosamine was the major hexosamine.

Table 1. *Chemical composition of the proteoglycan from human uterine cervix*

Composition of total proteoglycan	(nmol/mg)	(residues/ 1000 residues)
Aspartic acid	347	130
Hydroxyproline	Not detected	Not detected
Threonine	120	45
Serine	210	79
Glutamic acid	293	110
Proline	206	77
Glycine	228	85
Alanine	142	53
Valine	132	50
Methionine	8	3
Isoleucine	115	43
Leucine	325	122
Tyrosine	53	20
Phenylalanine	89	33
Histidine	66	25
Lysine	183	68
Arginine	109	41
Cysteine	45	17
Composition of total proteoglycan	(% dry wt.)	
Protein (amino acid analyser)	29	
Protein (Lowry method)	41	
Hexosamine	15.4	
Uronic acid	10.5	
Water	12	
Galactosamine/hexosamine ratio	0.85	
Carbohydrate side group	(% of total hexosamine)	
Oligosaccharide	9	
Glycosaminoglycan	91	
Neutral sugars in the oligosaccharides	(nmol/mg of proteoglycan)	
Xylose	5	
Mannose	9	
Galactose	9	
Glucose	4	

On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis the glycoconjugate behaved as a polydisperse material with an apparent molecular weight of 106000 (detection by periodate/Schiff or Kenacid staining). The protein core, obtained after chondroitinase ABC digestion, gave a sharp single band corresponding to a molecular weight of 47000. This proteoglycan nature was further confirmed by gel chromatography on Sepharose CL-6B (Fig. 5), where $K_{av.}$ of the proteoglycan changed from 0.32 before digestion to 0.44 after digestion by chondroitinase ABC.

There is a discrepancy between the protein content (29–42%) and the apparent molecular size of the protein core as estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (47000). Heinegård *et al.* (1981) have shown that the protein core isolated by chondroitinase ABC digestion from a small proteoglycan from cartilage

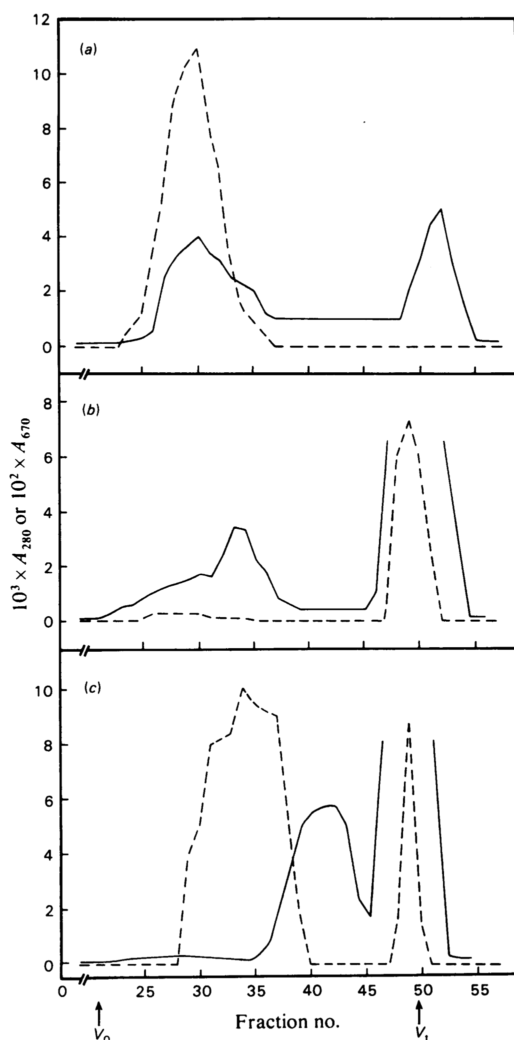

Fig. 5. *Gel chromatography on Sepharose CL-6B of* (*a*) *the purified proteoglycan,* (*b*) *a chondroitinase ABC digest of the proteoglycan and* (*c*) *a papain digest of the proteoglycan*

The column (60 cm × 0.5 cm) was eluted with 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. The eluent monitored continuously for A_{280} (——) with an LKB 2138 Uvicord instrument, and fractions (0.7 ml) were analysed for uronic acid (A_{670}, orcinol method, ----). V_0, Void volume; V_t, total volume.

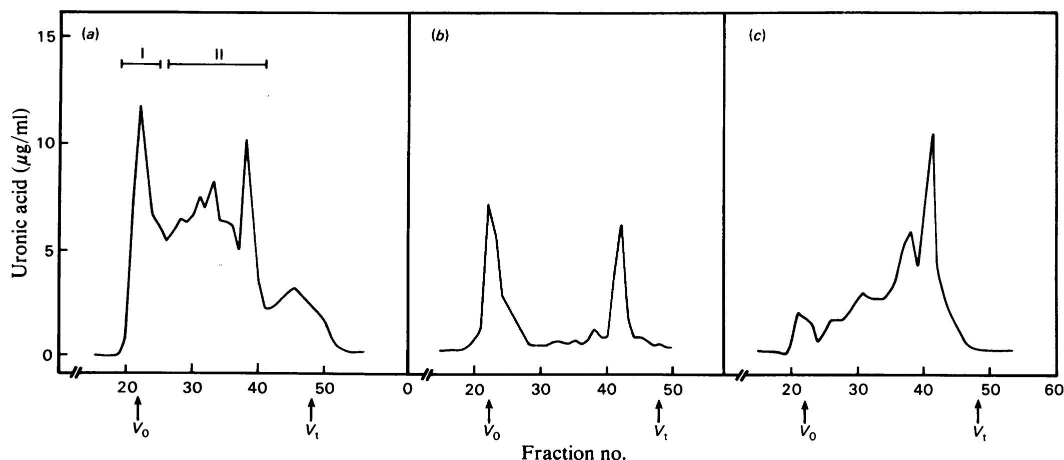

Fig. 6. *Gel chromatography on Sephadex G-50 of degradation products derived from the dermatan sulphate side chains*
Glycosaminoglycan side chains were degraded by periodate oxidation/alkaline elimination (*a*). The excluded (fraction I) and the partially included degradation (fraction II) products were separately digested with chondroitinase AC and re-chromatographed on the same column. Results are shown in (*b*) (fraction I) and (*c*) (fraction II). Column size was 5 mm × 1500 mm; eluent was 0.2 M-pyridine/acetate buffer, pH 5.0; analysis for uronic acid was by the carbazole method. V_0, Void volume; V_t, total volume.

has a lower apparent free electrophoretic mobility than do reference proteins. This may at least partly be due to the presence of oligosaccharides.

After DEAE-cellulose chromatography, 9% of the hexosamine of a papain digest was not retained. These residues are probably constituents of oligosaccharides of the proteoglycan.

In the glycosaminoglycan portion the hexosamines were exclusively galactosamine. The $K_{av.}$ of the polysaccharide was 0.45 on gel chromatography on Sepharose CL-6B, suggesting a molecular weight of roughly 25000 (Wasteson, 1971). Selective periodate oxidation of L-iduronic acid residues in the glycan side chains followed by scission in alkali and gel chromatography afforded the elution profile in Fig. 6. The major part of the degradation products was included on Sephadex G-50, which excludes polymers containing seven disaccharide residues per molecule. The fragments in the void volume (fraction I) contained an appreciable quantity of chondroitinase AC-resistant material composed of blocks of *O*-sulphated L-iduronic acid residues (Fig. 6*b*). Most of the material was, however, degraded to disaccharides containing D-glucuronic acid. The included fragments (fraction II) were also further degraded by chondroitinase AC.

The results indicate that the glycosaminoglycan is a dermatan sulphate with blocks of L-iduronic acid, D-glucuronic acid and L-iduronic acid *O*-sulphate. This 'fingerprint' of the dermatan sulphate resembles that from a similar preparation from bovine sclera, proteoglycan II (Cöster & Fransson, 1981).

The oligosaccharides contained, in addition to hexosamine, equal amounts of mannose and galactose. It is likely that the proteoglycans contain some linkage regions not further substituted with dermatan sulphate side chains in addition to mannose- and glucosamine-rich oligosaccharides.

General discussion

In addition to the 90% of connective tissue, the human uterine cervix contains smooth muscle, blood vessels and mucus-filled crypts, which extend from the epithelium deep into the tissue. It is, however, likely that the dermatan sulphate proteoglycan originates from the connective tissue, as mucus does not contain dermatan sulphate proteoglycan. Furthermore, the dermatan sulphate concentration is about the same as that in biopsy samples from which the mucosa has been carefully removed (Uldbjerg *et al.*, 1982*a*), and it is about the same as that in a pure connective tissue such as sclera (Cöster & Fransson, 1981). Blood vessels are probably not the source either, as the vascularization of the cervix increases during pregnancy, when the dermatan sulphate concentration remains constant or decreases (Shimizu *et al.*, 1980; Golichowski *et al.*, 1980).

The uterine cervix contains heparan sulphate but in lower concentration than dermatan sulphate

(Shimizu *et al.*, 1980; Golichowski *et al.*, 1980). The absence of heparan sulphate after the extraction procedure used in the present work may be explained if it is localized in such a way, for example in blood vessels (Höök *et al.*, 1974) and on cell surfaces, that it is difficult to extract.

The dermatan sulphate proteoglycan is similar to the smaller of those obtained from sclera (Cöster & Fransson, 1981). These proteoglycans are small, with weight-average molecular weight 90000, and contain a protein core that after chondroitinase ABC treatment has an apparent molecular weight, obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, of approx. 47000. This leaves room for one, two or three polysaccharide side chains per molecule. Furthermore, the amino acid compositions of the cores are also very similar, and contain aspartate, glutamate and leucine as dominating residues, with serine being present in considerably smaller amounts. It should be noted that the size of the proteoglycan and the characteristics of the core are similar to those for proteoglycans isolated from cartilage (Heinegård *et al.*, 1981). It may therefore be suggested that small proteoglycans are common for many tissues, since they contain very similar protein cores and numbers of side chains. The structure of the polysaccharide side chains varies, depending on the activity of the uronosyl epimerase in the various tissues. The chemical composition of the cervical dermatan sulphate seems to change during pregnancy (Shimizu *et al.*, 1980; Golichowski *et al.*, 1980). Studies of the proteoglycans have, however, not been published.

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